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APPLICATION NUMBER: *60/021,940*
FILING DATE: *July 17, 1996*

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

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PATENT APPLICATION SERIAL NO. 60/021940

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By: _____
Name: Darrell Whitaker

REQUEST FOR PROVISIONAL APPLICATION UNDER 37 C.F.R. § 1.53(b)(2)

Assistant Commissioner for Patents
Washington, DC 20231

Dear Sir:

This is a request for filing a Provisional application for patent under 37 CFR § 1.53(b)(2) entitled METHODS FOR REGULATING NICOTINE METABOLISM by the following inventor(s):

Full Name Of Inventor	Family Name SELLERS 1-00	First Given Name Edward	Second Given Name M.
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Residence & Citizenship	City	State or Foreign Country	Country of Citizenship
Post Office Address	Post Office Address	City	State & Zip Code/Country

1. ☒ Enclosed is the Provisional application for patent as follows: 53 pages of specification, 9 claims, 1 page Abstract, and 27 sheets of drawings.

2. ☒ A Verified Statement that this filing is by a small entity (37 CFR 1.9, 1.27, 1.28) is attached.

3. ☒ Payment of Provisional filing fee under 37 C.F.R. § 1.16(k):
   - ☒ Attached is a check in the amount of $ 75.00.
   - ☐ Please charge Deposit Account No. 13-2725.
   - ☐ PAYMENT OF THE FILING FEE IS BEING DEFERRED.

4. ☒ The Commissioner is hereby authorized to charge any additional fees as set forth in 37 CFR §§ 1.16 to 1.18 which may be required by this paper or credit any overpayment to Account No. 13-2725.

5. ☐ Enclosed is an Assignment of the invention to , Recordation Form Cover Sheet and a check for $ to cover the Recordation Fee.

6. ☐ Also Enclosed:

7. ☐ The invention was made by the following agency of the United States Goernment or under a contract with the following agency of the United States Government:

8. ☒ Address all future communications to the **Attention of Douglas P. Mueller, Esq.** (may only be completed by attorney or agent of record) at the address below.

9. ☒ A return postcard is enclosed.

Respectfully submitted,

July 17, 1996
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Signature

TITLE: Methods for Regulating Nicotine Metabolism

INVENTORS: Edward M. Sellers and Rachel F. Tyndale

Title: Methods for Regulating Nicotine Metabolism

FIELD OF THE INVENTION

The invention relates to methods for regulating nicotine metabolism in an individual; compositions for regulating nicotine metabolism in an individual; and methods of treating conditions requiring regulation of nicotine metabolism in an individual.

BACKGROUND OF THE INVENTION

Nicotine is the primary alkaloid present in tobacco playing a crucial role in establishing and maintaining tobacco dependence. Several studies have shown that smokers adjust their smoking behaviour to try and maintain constant nicotine blood levels, and hence brain nicotine levels. Studies using different nicotine yield cigarettes (Finnegan et al., 1945), nicotine replacement therapy [Lucchesi et al. (intravenous infusion), 1967; Jarvik et al., 1970 (ingestion); Kaslowski et al., 1975; Russell et al., 1976; Ebert et al., 1984 (nicotine chewing gum); Levin et al., 1994 (nicotine patches)], nicotine blockade (Stolerman et al., 1973l Nemeth-Coslett et al., 1986; Rose et al., 1994), and alteration of urinary pH (Benowitz et al., 1983; 1985; Rosenburg et al., 1980), showed that nicotine intake can be regulated to avoid exceeding the blood nicotine concentration of typical smoking levels. The studies provide clear evidence that smoking behaviour is modified in smokers to regulate nicotine blood levels. Therefore, changes in nicotine clearance from the body, such as metabolic changes, can have a significant impact on smoking behaviour.

Nicotine and its metabolites have been extensively studied over the past few decades. Nicotine is, for the most part, metabolized in the liver (80%), and to a smaller extent in the lungs and kidneys (Schievelbein, 1982; Turner, 1975). The major metabolite of nicotine is cotinine (Benowitz et al., 1994). Nicotine is primarily metabolized to cotinine through a two step process (Figure 1). The first step in the process produces the intermediate, nicotine-Δ-1'(5') imminium ion (Peterson and Castagnoli, 1988, Williams *et al.*, 1990), which is then further oxidized through a cytosolic aldehyde oxidase reaction

in the presence f liver microsomes, O_2 and NADPH (Hill *et al.*, 1972; Peterson *et al.*, 1987; Brandage *et al.*, 1979; Gorrod *et al*, 1982).

The cytochrome P450 (CYP) system has been implicated in the metabolism of nicotine. Evidence for CYP involvement in nicotine metabolism has come from rat liver studies in which reconstituted purified CYPs, and specific antibodies were shown to inhibit nicotine metabolism. In particular, rat studies have shown that phenobarbital inducible CYPs (i.e. the CYPs; -2B1, -2B2, -2C6, and -3A2) are involved in nicotine metabolism (Nakayama *et al.*, 1982; Hibberd and Gorrod 1985; Foth *et al.*, 1990; Seaton *et al.*, 1991 and 1993). Of 12 human CYPs forms tested, CYP2B6 showed the highest nicotine oxidase activity while CYP2E1 and CYP2C9 showed intermediate levels (Flammang et al., 1992). McCracken et al., (1992), have shown that human CYP2B6 and CYP2D6 displayed high rates of nicotine to cotinine metabolism, whereas the catalytic activity of CYP2E1 towards nicotine is not detectable. The results concerning CYP2E1 and CYP2D6 are in disagreement with the findings of Flammang et al., (1992). Thus, there remains some ambiguity concerning the affinities of CYPs for nicotine.

The CYP2B proteins are expressed at low amounts in the liver (less than 5% of the total hepatic CYP content) in all animals and humans, but their levels can be highly induced by exposure to a number of diverse chemicals, including the prototypic CYP2B inducer phenobarbital (Ryan et al., 1990; Guengerich et al., 1982b). The human CYP2B6 enzyme is expressed at variable levels among different individuals. CYP2B6 has poor oxidation activity towards benzopyrene, 7-ethoxycoumarin, coumarin, ethoxyresorufin, pentoxyresorufin, ethylmorphine, benphetamine, and aniline (Mimura et al., 1993). Orphenadrine, an anti-parkinsonian agent, was found to be a specific inhibitor of CYP2B6 (Reidy et al., 1992; Chang et al., 1993).

cDNA studies have implicated CYP2A6 in nicotine metabolism (Flammang et al., 1992; McCracken et al., 1992). CYP2A6 also displays a genetic polymorphism whereby certain individuals contain an inactive enzyme (Daly et al., 1994). CYP2A6 is the predominant, if not the only, coumarin 7-hydroxylase in humans (Pearce et al., 1992). CYP2A6 catalyzes the

hydroxylation of coumarin a naturally occurring compound in plants and essential oils (Pelkonan et al., 1985; Raunio et al., 1988; Yamano et al., 1990; Pearce et al., 1992). In primates, such as humans and baboons, coumarin is metabolized to 7-hydroxycoumarin (~80%) (Cholerton et al., 1992; Shilling et al., 1969; Moran et al., 1987). However, in rodent species such as the rat, mouse and hamster, 3-hydroxycoumarin is the major metabolite (Shilling et al., 1969; Egan et al., 1990). Early experiments on coumarin 7-hydroxylase activity, in human liver microsomes, demonstrated marked inter-individual differences in the expression levels of CYP2A6 (Kapitulnik et al., 1977; Pelkonen et al., 1985). Variability was also found in levels of expression of CYP2A6 mRNA in human livers (Miles et al., 1990; Yamano et al., 1990; Yun et al., 1991). In particular, CYP2A6 protein levels in human liver microsomes varied by over 100 fold (Yun et al., 1991). CYP2A6 also has been found to metabolize several procarcinogens such as NNK (Crespi et al., 1991), aflaxtoxin B1 (Yun et al., 1991); hexamethylphosphoramide (Ding et al., 1988), and nitrosodimethylamine (Davies et al., 1989; Fernandez et al., 1995).

SUMMARY OF THE INVENTION

The present inventors have found that variation in nicotine metabolism among individuals is due to variable expression of CYP2A6, and not CYP2D6. CYP2A6 has been shown to be the major nicotine metabolizing enzyme in human livers. Coumarin, a specific CYP2A6 substrate, was found to specifically and selectively inhibit nicotine metabolism to cotinine by 84% ± 11% in test livers, and addition of orphenadrine enhanced the inhibition. A monoclonal antibody raised against CYP2A6 also inhibited cotinine formation. The amount of CYP2A6, as determined by Western blots, was highly correlated to V_{max} ($r = 0.83$, $p < 0.001$), and to inhibition by coumarin ($r = 0.80$, $p < 0.001$). The data indicate that variability in CYP2A6 expression results in inter-individual variation in nicotine metabolism, which in turn, can have behavioral consequences such as smoking more or less cigarettes. Therefore, selective and specific inhibitors of CYP2A6 can be used to regulate nicotine

metabolism, and in particular substantially decrease nicotine metabolism, thereby affecting tobacco use.

Broadly stated the present invention relates to a method of regulating nicotine metabolism in an individual comprising selectively inhibiting CYP2A6. Inhibition of CYP2A6 may be achieved using one or more of the following (i) substances which inhibit CYP2A6 activity; or (ii) substances which inhibit transcription and/or translation of the gene encoding CYP2A6. CYP2A6 may also be selectively inhibited by interfering with the transcription or translation of the gene encoding CYP2A6 using gene transfer methods.

The present invention also provides a method of screening for a substance that regulates nicotine metabolism to cotinine in an individual comprising assaying for a substance which selectively (i) inhibits CYP2A6 activity; or (ii) inhibits transcription and/or translation of the gene encoding CYP2A6.

The invention further provides a pharmaceutical composition for use in treating a condition requiring regulation of nicotine metabolism to cotinine comprising an effective amount of a substance which selectively inhibits CYP2A6, and/or a pharmaceutically acceptable carrier, diluent, or excipient. A method is also provided for treating a condition requiring regulation of nicotine metabolism to cotinine in an individual comprising administering to the individual an effective amount of a substance which selectively inhibits CYP2A6.

CPY2B6 inhibitors may also be used in combination with inhibitors of CPY2A6 to provide an enhanced inhibitory effect. Therefore, the present invention provides a method for enhancing inhibition of nicotine metabolism by a CYP2A6 inhibitor in an individual comprising administering to the individual an effective amount of a substance which selectively inhibits CYP2A6, and an effective amount of an inhibitor of CPY2B6. A pharmaceutical composition for use in treating a condition requiring regulation of nicotine metabolism to cotinine is also provided comprising an effective amount of a substance which selectively inhibits CYP2A6, an effective amount of an inhibitor of CPY2B6, and/or a pharmaceutically

acceptable carrier, diluent, or excipient. Further, a method for treating a condition requiring regulation of nicotine metabolism to cotinine in an individual is provided comprising administering to the individual an effective amount of a substance which selectively inhibits CYP2A6, and an effective amount of an inhibitor of CPY2B6.

The pharmaceutical compositions and methods may be used to diminish a subjects desire for nicotine and thereby can be used to alter tobacco use.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

DESCRIPTION OF THE DRAWINGS

The invention will be better understood with reference to the drawings in which:

Figure 1 shows the two step conversion of nicotine to cotinine;

Figure 2A shows the amino acid and nucleotide sequence for cytochrome CYP2A6;

Figure 2B shows the mRNA sequence for cytochrome CYP2B6;

Figure 3 shows the protein-time curves of cotinine production from 100 μM nicotine in the presence of 20 μl rat cytosol by K20 human microsomes;

Figure 4 shows sample Michaelis-Menten curves, with inset Eddie-Hofstee plots, of nicotine to cotinine metabolism where (A) is a graph displaying single enzyme kinetics for the L29 human liver and (B) displays multiple enzyme site kinetics for the L30 human liver;

Figure 5 is a bar graph showing apparent K_m values of nicotine to cotinine metabolism by 30 human liver microsomes;

Figure 6 is a bar graph showing apparent V_{max} values of nicotine to cotinine metabolism by 30 human liver microsomes;

Figure 7 shows antibody activity (Gentest Corp.); where (A) is a graph of inhibition of coumarin oxidation by MAB-2A6 antibody and (B) is a graph of inhibition of testosterone 16β-hydroxylation (CYP2B1), and lidocaine methyl-hydroxylation (CYP2B2) by anti-CYP2B1 antibody;

Figure 8 shows Western blots of increasing concentrations of L64 microsomal protein (above) with respective densities plotted to show linearity of analysis (below);

Figure 9 is a bar graph showing coumarin (150 μM) inhibition of cotinine formation by 30 human liver microsomes;

Figure 10 is a bar graph showing percent inhibition of nicotine (100 μM) to cotinine metabolism by 150 μM coumarin by 30 human liver microsomes;

Figure 11 is a Dixon plot of coumarin inhibition of cotinine formation by K27 liver microsomes;

Figure 12 is a bar graph showing the effects of 10 μM and 100 μM concentrations of coumarin analogs on inhibiting cotinine formation by K27 human liver microsomes;

Figure 13 is a graph showing the effects of MAB-2A6 on nicotine (100 μM) to cotinine metabolism by K27 human liver microsomes;

Figure 14 shows Western blots of 30 human liver microsomes; each blot is accompanied by a 15, 30, 75 and 100 μg lanes of L64 microsomal protein such that individual blots can be compared;

Figure 15 is a graph showing the correlation between nicotine to cotinine V_{max} values and the amount of immunoreactive CYP2A6 by 30 human livers;

Figure 16 is a graph showing the correlation between CYP2A6 mediated cotinine formation and the amount of immunoreactive CYP2A6 by 30 human livers;

Figure 17 is a graph showing the correlation between nicotine to cotinine V_{max}/K_m values and the am unt of immunoreactive CYP2A6 by 30 human livers;

Figure 18 is a bar graph showing orphenadrine (150 μM) inhibition of cotinine formation by 30 human liver microsomes;

Figure 19 is a bar graph showing the percent inhibition of cotinine formation by orphenadrine (150 μM) using 30 human liver microsomes;

Figure 20 is a graph showing the effects of anti-rat CYP2B1 on cotinine formation by K27 human liver microsomes;

Figure 21 is a bar graph showing coumarin and orphenadrine (150 μM each) inhibition on nicotine metabolism by 30 human liver microsomes; and

Figure 22 is a bar graph showing troleandomycin (150 μM) inhibition of cotinine formation by 30 human liver microsomes; and

Figure 23 shows chemical structures of some representive CYP2A6 inhibitors.

DETAILED DESCRIPTION OF THE INVENTION

1. Method of Regulating Nicotine Metabolism in a Subject

As hereinbefore mentioned, the present invention relates to a method of regulating nicotine metabolism to cotinine in an individual comprising selectively inhibiting CYP2A6. Inhibition of CYP2A6 may be achieved using one or more of the following (i) substances which inhibit CYP2A6 activity; or (ii) substances which inhibit transcription and/or translation of the gene encoding CYP2A6.

Substances which inhibit CPY2A6 activity include substances which specifically bind to CYP2A6 and thereby inhibit its activity. Examples of such substances include antibodies which are specific for CPY2A6 including for example, the monoclonal antibody described by Pearce, R., et al, 1992., and commercially available antibodies such as MAB2A6 and anti-rat CYP2E1 sold by Gentest Corporation, Woburn, Mass., U.S.A., (Catalogue Nos. A112, A106, and 219221, respectively); and XenoTech 2A6 sold by XenoTech LLC, Kansas City, KS, U.S.A.

Substances which inhibit CYP2A6 activity also include substances having a lactone structure with a carbonyl oxygen. Examples f such substances include coumarin (The Merck Index, Eleventh Edition Budavari, S., ed. Merck & C . Inc., 1989, N . 2563), furanocoumarin, bishydroxycoumarin, methoxsalen (The Merck Index, No. 5911), imperatorin (The Merck Index, No. 4839), psoralen (The Merck Index, No. 7944), α-naphthoflavone, isopimpinellin, β-naphthoflavone, bergapten (The Merck Index, No. 1173), sphondin, coumatetralyl (racumin), and (+)-cis-3,5-dimethyl-2-(3-pyridyl)-thiazolidim-4-one (SM-12502) (Nunoya, et al., JPET 277:768-774, 1996). See Figure 23 for the chemical structures of some representative inhibitors. Derivatives and analogs of these substances may also be used in the methods and compositions of the invention. By way of example, derivatives of coumarin include pharmaceutically acceptable salts, esters and complexes of coumarin including potassium and sodium salts, and amino acid, carbohydrate and fatty acid complexes. Suitable analogs of coumarin may be selected based upon their functional similarity to coumarin, including the ability to inhibit the metabolism of nicotine to cotinine by CYP2A6 with greater than 50% inhibition, and/or a Ki less than 300 μM. Examples of functional analogs of coumarin include 7-methoxycoumarin, 7-methylcoumarin, and 7-ethoxycoumarin. Analogs of coumarin may also be selected based upon their three dimensional structural similarity to coumarin i.e. the lactone/carbonyl structure.

Other substances which inhibit CPY2A6 and can be used in the methods and compositions of the invention include diethyldithiocarbamate, nicotine and analogs and derivatives thereof, N-nitrosodialkylamine, diethylnitrosamine, nitropyrene, menadione (The Merck Index, No. 5714), imidazole antimycotics, miconazole (The Merck Index, No. 6101), clotrimazole (The Merck Index, No. 2412), psoralen (The Merck Index, No. 7944), pilocarpine (The Merck Index, No. 7395), and non-toxic analogs and derivatives of hexamethylphosphoramide, 4-methylnitrosamine-3-pyridyl-1-butanol, aflatoxin B (The Merck Index, No. 168), N-nitrosodiethylamine (The

Merck Index, No. 6557), and N-nitrosodimethylamine (The Merck Index, No. 6558),.

Substances which inhibit CYP2A6 activity may also be identified using the screening methods described herein.

Substances which inhibit transcription and/or translation of the gene encoding CYP2A6 include a nucleic acid sequence encoding the CYP2A6 gene (see Figure 2A, GenBank Accession No. HSU22027) or parts thereof (e.g. the region which is about 20 nucleotides on either side of nucleotide 790 (ATG), and the splice sites 1237, 2115, 2499, 3207, 4257, 4873, 5577 and 6308), inverted relative to their normal orientation for transcription i.e. antisense CYP2A6 nucleic acid molecules. Such antisense nucleic acid molecules may be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed with CYP2A6 mRNA or the CYP2A6 gene. The antisense sequences may be produced biologically using an expression vector introduced into cells in the form of a recombinant plasmid, phagemid or attenuated virus in which antisense sequences are produced under the control of a high efficiency regulatory region, the activity of which may be determined by the cell type into which the vector is introduced.

A nucleic acid molecule containing the antisense sequences may be introduced into cells in a subject using conventional techniques, such as transformation, transfection, infection, and physical techniques such as electroporation or microinjection. Chemical methods such as coprecipitation and incorporation of DNA into liposomes may also be used to deliver antisense sequences. The molecules may also be delivered in the form of an aerosol or by lavage. Suitable vectors or cloning vehicles for transferring the nucleic acid molecules are known in the art. Examples of suitable vectors include retroviral vectors, adenoviral vectors, and DNA virus vectors.

The ability of a substance to selectively inhibit CYP2A6 and thus regulate nicotine metabolism to cotinine may be confirmed using the methods described herein for screening for an inhibitor.

In preferred embodiments of the invention the CYP2A6 inhibitor is coumarin, or a derivative or analog thereof.

CYP2A6 may also be selectively inhibited in the method of the invention by interfering with the transcription of the gene encoding CYP2A6 using gene transfer methods such as targeted gene mutagenesis using allelic replacement, insertional inactivation, or deletion formation. For example, allelic gene exchange using non-replicating or conditionally-replicating plasmids has been used widely for the mutagenesis of eukaryotes. Allelic exchange can be used to create a deletion of the CYP2A6 gene. Exemplary methods of making the alterations set forth above are disclosed by Sambrook et al (Molecular Cloning: A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory Press, 1989).

CPY2B6 inhibitors may also be used in combination with inhibitors of CPY2A6 to provide an enhanced inhibitory effect. Inhibitors of CYP2B6 include one or more of the following (i) substances which inhibit CYP2B6 activity; or (ii) substances which inhibit transcription and/or translation of the gene encoding CYP2B6. CPY2B6 inhibitors may also be used alone to inhibit nicotine metabolism in an individual.

Substances which inhibit CPY2B6 activity include substances which specifically bind to CYP2B6 and thereby inhibit its activity. Examples of such substances include antibodies which are specific for CPY2B6 including for example, commercially available antibodies such as anti- CYP2B6 sold by Gentest Corporation, Woburn, Mass., U.S.A.

Substances which inhibit CYP2B6 activity also include substances selected from phenylethyl amines, diphenylbarbiturates, diethyl substituted barbiturates and hydantoins. In particular, diphenhydramine and its derivatives, including orphenadrine (The Merck Index, No. 6831), and derivatives or analogs of orphenadrine, and other antihistamines, anticholinergic substances such as cholines and analogs and derivatives thereof may be used as CYP2B6 inhibitors in the methods and compositions of the invention.

Derivatives of rphenadrine which may be used in the methods and compositions of the invention include pharmaceutically acceptable salts, esters and complexes of orphenadrine including potassium and sodium salts, and amino acid, carbohydrate and fatty acid complexes. Suitable analogs of orphenadrine may be selected based upon their functional similarity to orphenadrine, including the ability to inhibit CPY2B6 with greater than 50% inhibition, and/or a Ki less than 300 μM. Analogs of orphenadrine may also be selected based upon their three dimensional structural similarity to orphenadrine.

Substances which inhibit transcription and/or translation of the gene encoding CYP2B6 include a nucleic acid sequence encoding the CYP2B6 gene (see Figure 2B, GenBank Accession No. HSP452B6 for the mRNA sequence of CYP2B6), or parts thereof (e.g. the region which is on either side of nucleotide 9 (ATG), and the sites 111, 274, 424, 585, 762, 904, 1092, and 1234 nt), inverted relative to their normal orientation for transcription i.e. antisense CYP2B6 nucleic acid molecules. Such antisense nucleic acid molecules may be produced and introduced into cells using conventional procedures as described herein.

CYP2B6 may also be selectively inhibited in a method of the invention by interfering with the transcription of the gene encoding CYP2B6 using conventional gene transfer methods as discussed herein.

In preferred embodiments of the invention the CYP2B6 inhibitor employed is orphenadrine and derivatives or analogs of orphenadrine.

An inhibitor of CYP2A6 or CYP2B6 may be targeted to the enzyme using antibodies specific to an epitope of the enzyme. For example, bispecific antibodies may be used to target an inhibitor. The bispecific antibodies contain a variable region of an antibody specific for at least one epitope of CYP2A6 or CYP2B6, and a variable region of a second antibody which is capable of binding to an inhibitor. The bispecific antibodies may be prepared by forming hybrid hybridomas, using procedures known in the art such as those disclosed in Staerz & Bevan, (1986, PNAS (USA) 83: 1453) and Staerz & Bevan, (1986,

Immunology Today, 7:241). Bispecific antibodies may also be constructed by chemical means using conventional procedures such as those described by Staerz et al., (1985, Nature, 314:628) and Perez et al., (1985 Nature 316:354), or by expression of recombinant immunoglobulin gene constructs.

The inhibitory activity of a particular substance identified herein or an analog or derivative thereof may be confirmed in experimental model systems, and in clinical studies, for example, the studies as outlined in Examples 3 to 6 herein.

2. Screening Methods

As hereinbefore mentioned, the present invention provides a method of screening for a substance that regulates nicotine metabolism to cotinine in an individual comprising assaying for a substance which selectively (i) inhibits CYP2A6 activity, or (ii) inhibits transcription and/or translation of the gene encoding CYP2A6.

In an embodiment of the method of the invention, a method is provided for screening for a substance that regulates nicotine metabolism to cotinine in a subject by inhibiting CYP2A6 activity comprising the steps of :

(a) reacting a substrate of CYP2A6 and CYP2A6, in the presence of a test substance, under conditions such that CYP2A6 is capable of converting the substrate into a reaction product;

(b) assaying for reaction product, unreacted substrate or unreacted CYP2A6;

(c) comparing to controls to determine if the test substance selectively inhibits CYP2A6 and thereby is capable of regulating nicotine metabolism in an individual.

Substrates of CYP2A6 which may be used in the method of the invention for example include nicotine and coumarin and analogs and derivatives thereof. The corresponding reaction products for nicotine and coumarin are cotinine, and 7-hydroxycoumarin, respectively.

CYP2A6 used in the method of the invention may be obtained from natural, recombinant, or commercial sources. For example CYP2A6 may be obtained by recombinant methods such as those described by Nesnow, S. et al.,

Mutation Research 1994; 324:93-102. Cells or liver mic[illegible]xpressing CYP2A6 may also be used in the method.

Conditions which permit the formation of a reaction product may be selected having regard to factors such as the nature and amounts of the test substance and the substrate.

The reaction product, unreacted substrate, or unreacted CYP2A6; may be isolated by conventional isolation techniques, for example, salting out, chromatography, electrophoresis, gel filtration, fractionation, absorption, polyacrylamide gel electrophoresis, agglutination, or combinations thereof.

To facilitate the assay of the reaction product, unreacted substrate, or unreacted CYP2A6; antibody against the reaction product or the substance, or a labelled CYP2A6 or substrate, or a labelled substance may be utilized. Antibodies, CYP2A6, substrate, or the substance may be labelled with a detectable marker such as a radioactive label, antigens that are recognized by a specific labelled antibody, fluorescent compounds, enzymes, antibodies specific for a labelled antigen, and chemiluminescent compounds.

The substrate used in the method of the invention may be insolubilized. For example, it may be bound to a suitable carrier. Examples of suitable carriers are agarose, cellulose, dextran, Sephadex, Sepharose, carboxymethyl cellulose polystyrene, filter paper, ion-exchange resin, plastic film, plastic tube, glass beads, polyamine-methyl vinyl-ether-maleic acid copolymer, amino acid copolymer, ethylene-maleic acid copolymer, nylon, silk, etc. The carrier may be in the shape of, for example, a tube, test plate, beads, disc, sphere etc.

The insolubilized CYP2A6, substrate, or substance may be prepared by reacting the material with a suitable insoluble carrier using known chemical or physical methods, for example, cyanogen bromide coupling.

In another embodiment of the invention, a method is provided for screening for a substance that regulates nicotine metabolism to cotinine in an individual by inhibiting transcription and/or translation of the gene encoding CYP2A6 comprising the steps of:

(a) culturing a host cell comprising a nucleic acid molecule containing a nuclei acid sequence encoding CYP2A6 and the necessary elements for the transcription or translation of the nucleic acid sequence, and optionally a reporter gene, in the presence of a test substance; and

(b) comparing the level of expression of CYP2A6, or the expression of the protein encoded by the reporter gene with a control cell transfected with a nucleic acid molecule in the absence of the test substance.

A host cell for use in the method of the invention may be prepared by transfecting a suitable host with a nucleic acid molecule comprising a nucleic acid sequence encoding CYP2A6. A nucleic acid sequence encoding CYP2A6 may be constructed having regard to the sequence of the CYP2A6 gene (Figure 2A) following procedures known in the art. Suitable transcription and translation elements may be derived from a variety of sources, including bacterial, fungal, viral, mammalian, or insect genes. Selection of appropriate transcription and translation elements is dependent on the host cell chosen, and may be readily accomplished by one of ordinary skill in the art. Examples of such elements include: a transcriptional promoter and enhancer or RNA polymerase binding sequence, a ribosomal binding sequence, including a translation initiation signal. Additionally, depending on the host cell chosen and the vector employed, other genetic elements, such as an origin of replication, additional DNA restriction sites, enhancers, and sequences conferring inducibility of transcription may be incorporated into the expression vector. It will also be appreciated that the necessary transcription and translation elements may be supplied by the native CYP2A6 gene and/or its flanking sequences.

Examples of reporter genes are genes encoding a protein such as β-galactosidase, chloramphenicol acetyltransferase, firefly luciferase, or an immunoglobulin or portion thereof such as the Fc portion of an immunoglobulin, preferably IgG. Transcription of the reporter gene is monitored by changes in the concentration of the reporter protein such as β-galactosidase, chloramphenicol acetyltransferase, or firefly luciferase. This

makes it possible to visualize and assay f r expression of CYP2A6 and in particular to determine the effect of a substance on expression of CYP2A6.

Suitable host cells include a wide variety of prokaryotic and eukaryotic host cells, including bacterial, mammalian, yeast or other fungi, viral, plant, or insect cells.

Protocols for the transfection of host cells are well known in the art (see, Sambrook et al. Molecular Cloning A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press, 1989, which is incorporated herein by reference). By way of example, Nanji M., et al., (1994) describe the expression of a cDNA encodir.g human CYP2A6 in a baculovirus system; Nesnow, S., et al. (1994) and Tiano H.F. et al, (1993) describe the expression of CYP2A6 from a retroviral vector in transformable C3H/10T1/2 mouse embryo fibroblasts; and Salonpaa, P., et al, (1993) describe the preparation of amphotropic recombinant retroviruses containing CYP2A6 using LXSN vector and PA317 packaging cells.

Host cells which are commercially available may also be used in the method of the invention. For example, the h2A3 and h2B6 cell lines available from Gentest Corporation are suitable for the screening methods of the invention.

The above mentioned methods of the invention may be used to identify negative regulators of nicotine metabolism to cotinine in brain and liver thereby affecting conditions requiring regulation of nicotine metabolism. Identification and isolation of such regulators will permit studies of the role of the regulators in the regulation of nicotine metabolism to cotinine and permit the development of substances which affect this role, such as functional or non-functional analogs of the regulators. It will be appreciated that such substances will be useful as pharmaceuticals to modulate nicotine metabolism to cotinine as discussed herein.

The inhibitory activity of the substances identified by the methods of the invention may be confirmed in experimental model systems, and in clinical studies, for example the studies as outlined in Examples 3 to 6 herein.

3. Compositions

Substances which inhibit nicotine metabolism to cotinine described in detail herein or substances identified using the methods of the invention which selectively inhibit CYP2A6 may be incorporated into pharmaceutical compositions. Therefore the invention provides a pharmaceutical composition for use in treating a condition requiring regulation of nicotine metabolism to cotinine comprising an effective amount of one or more substances which selectively inhibit CYP2A6, and/or a pharmaceutically acceptable carrier, diluent, or excipient. A method is also provided for treating a condition requiring regulation of nicotine metabolism to cotinine in a subject comprising administering to the subject an effective amount of one or more substances which selectively inhibit CYP2A6.

Conditions requiring regulation of nicotine metabolism to cotinine include nicotine use disorders i.e. dependent and non-dependent tobacco use, and nicotine-induced disorders i.e. withdrawal. The conditions may develop with the use of all forms of tobacco (e.g. cigarettes, chewing tobacco, snuff, pipes, and cigars) and with prescription medications (nicotine gum, nicotine patch, spray or other forms). In particular, the pharmaceutical compositions and treatment methods of the invention may be used to diminish a subjects desire to smoke and thereby alter smoking behaviour.

The compositions and treatment methods of the present invention by regulating nicotine metabolism in an individual are highly effective. The methods and compositions maintain the behavioral components of smoking and modify them by reducing nicotine metabolism to cotinine. An individual with reduced nicotine metabolism following administration of a composition of the present invention, will alter smoking behaviour and smoke exposure because of modification of nicotine requirements. The methods and compositions of the invention show patterns of reduction, more sustained abstinence, and lower tobacco smoke exposure than obtained with prior art methods in particular those using nicotine deprivation.

The behavioral component of smoking is particularly important in some groups of individuals, and thus the methods and compositions of the invention in modifying and maintaining behavioral components may be

particularly useful in reducing smoking in those individuals. For example, it has been found that behavioral components are significant in tobacco use by women. The present invention permits the d velopment of behavioral learning on an individual/or group basis.

The compositions and treatment methods of the invention are also particularly suited to regulate nicotine metabolism in individuals or populations having high levels of CYP2A6. For example, Caucasians in North America have high levels of CYP2A6. An individual or population having a high level of CYP2A6 can be identified using conventional methods for measuring CYP2A6.

The compositions and methods of the invention also have the advantage of individualization and flexibility in treatment duration. The compositions and treatment methods are particularly suitable for severely dependent individuals, previous treatment failures, individuals unable to accept the current approach of complete cessation, treatment/prevention of relapse, or concurrent treatment with other methods such as the nicotine patch. It is expected that the compositions and treatments of the invention will decrease the doses of nicotine patch needed and prolong the duration of its action.

The methods and compositions of the invention in treating individuals with nicotine use disorders and nicotine-induced disorders are also useful in the treatment and prophylaxis of diseases or conditions, including nicotine-related disorders such as opioid related disorders; proliferative diseases; cognitive, neurological or mental disorders; and other drug dependencies in the individuals. Examples of such underlying diseases or conditions include malignant disease, psychosis, schizophrenia, anxiety, depression, alcoholism, and opiate dependence.

The methods and compositions of the invention may also be used in the prophylaxis and treatment of individuals having a condition which requires a reduction in CYP2A6 or CYP2B6. For example, CYP2A6 is known to metabolize several procarcinogens such as NNK (Crespi et al., 1991), aflaxtoxin B1 (Yun et al., 1991); hexamethylphosphoramide (Ding et al., 1988), and

nitrosodimethylamine (Davies et al., 1989; Fernandez et al., 1995). Therefore, inhibitors of CYP2A6 may be useful in the prophylaxis and treatment of malignant diseases.

The pharmaceutical compositions of the invention contain substances which selectively inhibit CYP2A6 described in detail herein or substances identified using the methods of the invention. The active substances can be administered alone, but are generally administered with a pharmaceutical carrier etc. (see below), selected on the basis of the chosen route of administration and standard pharmaceutical practice.

The dosage administered will vary depending on the use and known factors such as the pharmacodynamic characteristics of the particular substance, and its mode and route of administration; age, health, and weight of the individual recipient; nature and extent of symptoms, kind of concurrent treatment, frequency of treatment, and the effect desired.

The individual recipient may be any type of mammal, but is preferably a human. Generally, the recipient is an individual having a CYP2A6 genotype and/or CYP2B6 genotype associated with an active form of the enzyme, preferably individuals having a CYP2A6 genotype associated with an active form of the enzyme. The determination of a CYP2A6 or CYP2B6 genotype and active CYP2A6 may be determined using procedures known in the art. For example, coumarin 7-hydroxylation has been used to measure CYP2A6 activity (Cholerton et al., 1992, J. Chromatogr. 575:325-330; and Rautio et al., 1992 Pharmaconetics 2:227-233). As discussed above, the methods and compositions of the invention may be preferably used in individuals or populations having high levels of CYP2A6, or in individuals where the behavioral components of smoking are significant.

For use in the treatment of conditions requiring regulation of nicotine metabolism to cotinine, by way of general guidance, a daily oral dosage of an active ingredient such as coumarin can be about 0.1 to 80 mg/kg of body weight, preferably 0.1 to 20, more preferably 0.2 to 3 mg/kg of body weight. Ordinarily a dose of 0.5 to 50 mg/kg of coumarin per day in divided doses one to multiple times a day, preferably up to four times per day, or in

sustained release f rm is effective to btain the desired results. In accordance with a particular regimen, coumarin is administered twice daily for one to four days. While standard interval dose administration may be used the compositions of the invention may be administered intermittently prior to high risk smoking times, e.g. early in the day and before the end of a working day.

The substances for the present invention can be administered for oral, topical, rectal, parenteral, local, inhalant or intracerebral use. In an embodiment of the invention, the substances are administered in intranasal form via topical use of suitable intranasal vehicles, or via transdermal routes, using forms of transdermal skin patches known to those of ordinary skill in that art. To be administered in the form of a transdermal delivery system, the dosage administration will be continuous rather than intermittent throughout the dosage regimen.

In the methods of the present invention, the substances described in detail herein and identified using the method of the invention form the active ingredient, and are typically administered in admixture with suitable pharmaceutical diluents, excipients, or carriers suitably selected with respect to the intended form of administration, that is, oral tablets, capsules, elixirs, syrups and the like, consistent with conventional pharmaceutical practices.

For example, for oral administration in the form of a tablet or capsule, the active substances can be combined with an oral, non-toxic, pharmaceutically acceptable, inert carrier such as lactose, starch, sucrose, glucose, methyl cellulose, magnesium stearate, dicalcium phosphate, calcium sulfate, mannitol, sorbitol and the like; for oral administration in liquid form, the oral active substances can be combined with any oral, non-toxic, pharmaceutically acceptable inert carrier such as ethanol, glycerol, water, and the like. Suitable binders, lubricants, disintegrating agents, and coloring agents can also be incorporated into the dosage form if desired or necessary. Suitable binders include starch, gelatin, natural sugars such as glucose or beta-lactose, corn sweeteners, natural and synthetic gums such as acacia, tragacanth, or sodium alginate, carboxymethylcellulose, polyethylene glycol, waxes, and the

like. Suitable lubricants used in these dosage forms include sodium oleate, sodium stearate, magnesium stearate, sodium benzoate, sodium acetate, sodium chl ride, and the like. Examples of disintegrators include starch, methyl cellulose, agar, bentonite, xanthan gum, and the like.

Gelatin capsules may contain the active substance and powdered carriers, such as lactose, starch, cellulose derivatives, magnesium stearate, stearic acid, and the like. Similar carriers and diluents may be used to make compressed tablets. Tablets and capsules can be manufactured as sustained release products to provide for continuous release of active ingredients over a period of time. Compressed tablets can be sugar coated or film coated to mask any unpleasant taste and protect the tablet from the atmosphere, or enteric coated for selective disintegration in the gastrointestinal tract. Liquid dosage forms for oral administration may contain coloring and flavoring agents to increase patient acceptance.

Water, a suitable oil, saline, aqueous dextrose, and related sugar solutions and glycols such as propylene glycol or polyethylene glycols, may be used as carriers for parenteral solutions. Such solutions also preferably contain a water soluble salt of the active ingredient, suitable stabilizing agents, and if necessary, buffer substances. Suitable stabilizing agents include antioxidizing agents such as sodium bisulfate, sodium sulfite, or ascorbic acid, either alone or combined, citric acid and its salts and sodium EDTA. Parenteral solutions may also contain preservatives, such as benzalkonium chloride, methyl- or propyl-paraben, and chlorobutanol.

The substances described in detail herein and identified using the methods of the invention can also be administered in the form of liposome delivery systems, such as small unilamellar vesicles, large unilamellar vesicles, and multilamellar vesicles. Liposomes can be formed from a variety of phospholipids, such as cholesterol, stearylamine, or phosphatidylcholines.

Substances described in detail herein and identified using the methods of the invention may also be coupled with soluble polymers which are targetable drug carriers. Examples of such polymers include polyvinylpyrrolidone, pyran copolymer,polyhydroxypropylmethacrylamide-

phenol, polyhydroxyethylaspartamidephenol, or polyethyleneoxide-polylysine substituted with palmitoyl residues. The substances may also be coupled to biodegradable polymers useful in achieving controlled release of a drug. Suitable polymers include polylactic acid, polyglycolic acid, copolymers of polylactic and polyglycolic acid, polyepsilon caprolactone, polyhydroxy butyric acid, polyorthoesters, polyacetals, polydihydropyrans, polycyanoacylates, and crosslinked or amphipathic block copolymers of hydrogels.

Pharmaceutical compositions suitable for administration contain about 1 milligram to 1500 milligrams of active substance per unit. In these pharmaceutical compositions, the active ingredient will ordinarily be present in an amount of about 0.5-95% by weight based on the total weight of the composition.

Suitable pharmaceutical carriers and methods of preparing pharmaceutical dosage forms are described in Remington's Pharmaceutical Sciences, Mack Publishing Company, a standard reference text in this field.

More than one substance described in detail herein or identified using the methods of the invention may be used to regulate metabolism of nicotine to cotinine. In such cases the substances can be administered by any conventional means available for the use in conjunction with pharmaceuticals, either as individual separate dosage units administered simultaneously or concurrently, or in a physical combination of each component therapeutic agent in a single or combined dosage unit. The active agents can be administered alone, but are generally administered with a pharmaceutical carrier selected on the basis of the chosen route of administration and standard pharmaceutical practice as described herein.

The combination of an CYP2A6 inhibitor e.g. coumarin, and a CYP2B6 inhibitor e.g. orphenadrine enhances inhibition of nicotine metabolism to cotinine. Thus, a preferred embodiment of the invention provides a method for treating conditions requiring regulating nicotine metabolism to cotinine comprising administering an effective amount of an CYP2A6 inhibitor and an effective amount of a CYP2B6 inhibitor to selectively inhibit nicotine metabolism to cotinine. In a preferred embodiment of the

invention, the CYP2A6 inhibitor is coumarin or an analog r derivative thereof, and the CYP2B6 inhibitor is orphenadrine, or an analog or derivative thereof. The inhibitors may be administered concurrently, separately or sequentially. The doses of the CYP2A6 inhibitor and the CYP2B6 inhibitor are each selected so that each inhibitor alone would not show a full effect. The effective doses are those which are approximately the minimum doses adequate for enhanced inhibition of nicotine metabolism to cotinine.

Pharmaceutical compositions containing combinations of CYP2A6 and CYP2B6 inhibitors may be prepared, and administered as described herein for the compositions containing CYP2A6 inhibitors. The pharmaceutical compositions preferably contain coumarin or an analog or derivative thereof, and orphenadrine, or an analog or derivative thereof, in concentrations of 1 to 1500 mg, and 25 to 400 mg, respectively.

The recognition by the present inventor that CYP2A6 is the major nicotine metabolizing enzyme in human livers suggests that the enzyme can be assayed in an individual to determine the individual's risk of developing tobacco dependence. Determination of CYP2A6 levels may also be used to select and monitor in an individual appropriate conventional nicotine replacement therapies such as the nicotine patch and nicotine gum. It is unlikely that nicotine replacement therapies will have a high success outcome if an individual has high levels of CYP2A6. Conversely, if an individual has very low levels of CYP2A6, administering nicotine at high dosages will likely result in increased toxicity, and side effects.

The following non-limiting examples are illustrative of the present invention:

EXAMPLE 1

Role of CYP2D6 in Nicotine Metabolism

The following materials and methods were utilized in the investigations outlined in example 1:

MATERIALS AND METHODS

Biological Samples

Human livers. The characteristics and sources of the K series livers used in this study were described previously (Campbell et al., 1987; Tyndale et al., 1989). The K series livers were obtained from Dr. T. Inaba, University of Toronto. The L series livers were obtained from Dr. E. Roberts from the Hospital for Sick Children (Toronto, ON, Canada), and consisted of partial livers obtained from liver donors.

CYP2D6 yeast. Microsomal preparations of CYP2D6 expressed in yeast (aH22/pelt1 cells) and control yeast (AH22/pMA91 cells) were provided by Dr. M.S. Lennard, University of Sheffield, U.K.. Immunochemical and catalytic assays have indicated that cytochrome P450 was undetectable in microsomes prepared from the control yeast, and that the enzyme activity in microsomes prepared from CYP2D6 expressing yeast was predominantly due to CYP2D6 (Ellis et al., 1992). Microsomal protein concentrations were determined by the BSA assay kit (Pierce Chemical Co., Rockford, IL, USA).

Drugs and Chemicals. Dextromethorphan hydrobromide, (S)-nicotine, (S)-cotinine, quinidine, ketamine, cumene hydroperoxide, and NADPH were obtained from Sigma Co. (St. Louis, MO, USA). Dextrorphan, methoxymorphinan, and hydroxymorphinan were provided by Hoffmann-La Roche Inc., Nutley, (N.J., USA). Budipine was obtained from Byk Gulden Pharmazeutika, Konstanz, Germany.

Microsome Preparation. The partial livers (~2 grams) from 30 humans were thawed on ice, then minced in two volumes of cold 1.15% KCl. The samples were homogenized by applying ten strokes of a Teflon pestle powered by a Black and Decker electric drill. Each liver homogenate was then subjected to a centrifugation of 9000 g for 20 min. at 4°C in a Sorvall RC2-B. The supernatant, which contains cytosol and microsomes, was decanted and centrifuged at 100,000 g for 60 min. at 4°C in a Sorvall Combi Plus Ultraspeed Centrifuge. The resulting microsomal pellet was resuspended in 1.15% KCl and centrifuged again at 100,000 g for 60 min. at 4°C for further purification. The microsomal pellet was resuspended in a 2:1 vol/wt solution of 1.15% KCl and stored in a Forma Scientific freezer at -70°C.

Protein Determination. Protein concentration of the microsomal samples were determined with a Pierce BCA pr tein assay kit (Pierce Chemical Co., Rockford, IL, USA), using the bovine serum albumin standard (BSA) solution provided. Samples, in duplicate, were diluted with H_2O to a concentration in the range of the BSA standards. 100 µl of each sample, or BSA standard, was added to 2 mls of Pierce Working Reagent. This reagent solution contained 50 parts Reagent A to 1 part Reagent B. Samples were vortexed and then incubated in a shaking water bath for 30 min. at 37°C. Absorbence of each sample was then measured at 562 nm against a blank vial.

Analytical Methods for Dextromethorphan assay. Incubation conditions of this assay were adapted from those of Otton et al., (1983).

Dextromethorphan to dextrorphan kinetics: Dextromethorphan to dextrorphan kinetics was measured as a function of protein concentration and time. Dextromethorphan at a concentration of 5 µM was incubated with 0.025, 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 mg protein/ml, with 0.8 mM NADPH in 0.2 M phosphate buffer (pH 7.4). The incubation mixture was comprised of 125 µl phosphate buffer (pH 7.4), 50 µl microsomal protein, 50 µl dextromethorphan, and 25 µl NADPH for a total volume of 250 µl. Incubations were carried out at 37°C for 30 min. in a shaking water bath, and were terminated with the addition of 10 µl perchloric acid. Budipine was used as an internal standard. Samples were then centrifuged at 3000 rpm for 5 min., and 30 µl of the supernatant was analyzed by HPLC. Results revealed that dextrorphan production was linear from 0.025 to 0.5 mg protein/ml throughout a 30 min. incubation. Apparent K_m and V_{max} values were determined by incubating dextromethorphan at 1, 2.5, 5, 10, 50, and 75 µM concentrations in duplicates, with a microsomal protein concentration of 0.125 mg/ml, for 30 minutes at 37°C.

HPLC: The HPLC system (Hewlett Packard) consists of a 1050 series pump and autosampler, connected to a HP 3396 series II integrator. A CSC-Spherisorb-Phenyl (5 um, 4.6 mm x 25 cm) column and a mobile phase consisting of 10

mM potassium phosphate buffer c ntaining 1 mM Heptanesulfonic acid, pH 3.8, and acetonitrile (80:20 v/v) was used, with a flow rate set at 1.7 ml/min. Dextromethorphan and the various metabolites in the incubation samples were measured as described by Broley et al. (1989), except that the excitation and emission wavelengths were set at 195 nm and 280 nm, respectively for a higher sensitivity. Dextrorphan calibration curves were linear from 0 to 120 pmoles, with the lowest detectable level of 5 pmoles for dextrorphan. The coefficient of variation within-day was 2.7 and 2.0 % (n=5), for 0.25 and 0.5 nmoles/ml injections of dextrorphan respectively. The coefficient of variation between-day was 6.5 and 9.6 % (n=6), for 0.125 and 0.5 nmoles/ml concentrations of dextrorphan respectively.

Analytical Methods for the Nicotine assay

Incubation: Microsomes were removed from a -70°C freezer and thawed on ice. Incubation mixtures generally contained 100 µl (S)-nicotine, 100 µl NADPH (1mM final), 200 µl human liver microsomes (0.5 mg/ml), 20 µl Wistar rat liver cytosol, 200 µl potassium phosphate buffer (pH 7.4, 40 µM final), diluted to a 1 ml final volume with 1.15% KCl. The reaction was initiated with the addition of NADPH and placing the samples at 37°C. The mixtures were incubated in a polypropylene conical 10 ml tubes then placed in a Precision Scientific Shaker Bath (Model 50) at 37°C for 45 minutes. The reaction was stopped by adding 100 µl of 20% Na_2CO_3 (pH 11.4). Nicotine to cotinine kinetic studies were performed by incubating 1, 5, 10, 50, 100, and 200 µM (S)-nicotine with 0.5 mg/ml microsomal protein for 45 min, in the presence of 20 µl rat liver cytosol, 1 mM NADPH, in 40 mM phosphate buffer (pH 7.4). The reaction was started by adding NADPH. The incubation mixture comprised of kinetic parameters were calculated by use of computer program Enzfitter (Robin J. Leatherbarrow, 1987). The data were fit by one-site Michaelis-Menten rate equation.

Extraction: After basification with Na_2CO_3, 10 µl of ketamine (the internal standard) was added to each sample. Ethyl acetate (3 mls) was added for

extraction purposes. The samples were vortexed vigorously for 5 min., followed by centrifugati n for 5 min. at 3000 rpm in an GLC-2B centrifuge. The organic layer (top) was pipetted to a separate 10 ml conical tube, which contained 400 µl of 0.01 N HCl. The samples were vortexed again for 5 min. and centrifuged at 3000 rpm for 5 min. The organic layer was then discarded, and the remaining aqueous layer was dried under nitrogen at 37°C for 30 min. to remove any remaining organic solvent. 30 µl of each sample was then subjected to High Performance Liquid Chromatography (HPLC) analysis.

HPLC: Separation of nicotine and metabolites was achieved by using a CSC-Spherisorb-Hexyl column (15 x .46 cm) and a mobile phase consisting of 20% acetonitrile and 80% 20 mM potassium phosphate, pH 4.6, containing 1 mM octanesulfonic acid was used. The separation was performed with isocratic elution at a flow rate of 1 ml/min. The retention times for cotinine, nicotine and ketamine were 3.5, 4.2, and 7.0 minutes respectively. The minimum detectable limit in the system was 300 pmoles of cotinine per ml of incubation mixture. Within and between day variations were found to be below 10% (n=6). The HPLC system consisted of a Hewlett Packard 1090 solvent delivery system linked to a 1050 series UV detector. The UV detector was set at 210 nm to optimize for cotinine detection.

Data Analysis. The UV absorbence data was transferred to a Hewlett-Packard Chemstation. The three peaks of interest were cotinine, nicotine, and ketamine (internal standard). The heights of the respective peaks were used to determine peak height ratios. Specifically, the cotinine peak height ratio was determined by measuring the ratio between the height of the cotinine peak to the ketamine peak height.

$$\text{Peak Height Ratio (PHR)} = \frac{\text{cotinine peak height}}{\text{ketamine peak height}}$$

The peak height ratios were used to analyze the relative amount of cotinine production, and to determine the specific concentration of cotinine present in

each sample incubation. This was achieved by producing standard curves during each session of data collection.

Standard curves were produced by injecting various concentrations of cotinine into the HPLC. Cotinine amounts typically included 1.25, 2.5, 5.0, and 10.0 nmole concentrations. 10 µl of ketamine, at a concentration of 0.25 mg/ml, was added to each sample. The standard curve enabled any given PHR obtained from a given sample to be converted to its respective concentration of cotinine in nmoles.

Within-day and Between-day variations. The within-day variation and between day variation of the assay were calculated for two concentrations of cotinine. Standard solutions contained 2.5 nmoles and 5.0 nmoles of cotinine per ml of incubation mixture. Samples contained 40 mM phosphate buffer, and 1.15 % KCl. They were subjected to similar extraction and evaporation procedures as mentioned above. For cotinine concentrations of 2.5 nmoles and 5.0 nmoles/ml the within day coefficients of variations were 3.1% and 2.3% respectively. The between-day variation were 7.2 % and 8.4%. A coefficient of variation (CV) less than 10% was deemed acceptable.

Cytosol assay. Cytosolic fractions from livers of 4 male Wistar rats were used as the source of aldehyde oxidase. Since the metabolism of nicotine to cotinine is a two step reaction involving an iminium ion intermediate, it was necessary to make the cytochrome P450 oxidation of nicotine the rate determining step. This was performed by adding excess aldehyde oxidase. Cotinine production increases and then plateaus with the addition of increasing amounts of rat cytosol. It was determined that 20 µl of cytosol would be used as the source of aldehyde oxidase. The rat cytosol had no intrinsic nicotine oxidase activity.

Protein - time assay. Protein concentrations of 0.125, 0.25, 0.5, and 1 mg protein/ml from the K20 liver microsome sample were incubated at 37°C, with 20 µl rat liver cytosol, 1 mM NADPH, in 40 mM phosphate buffer (pH 7.4) across several time intervals. Results are presented in Figure 3. These results show that cotinine formation is linear at a protein concentration of 0.125 to 0.5

mg/ml for a 45 min. incubati n. C tinine formation was also dependent on NADPH concentrations. A NADPH concentration of 1 mM was determined t be optimal for the above experimental conditions.

Quinidine and coumarin inhibition of cotinine formation. Nicotine was incubated with 0.5 mg/ml microsomal protein from K20 human liver. Incubations included 1 mM NADPH, 20 µl rat liver cytosol, 40 mM phosphate buffer (pH 7.4), and were carried out for 45 minutes at 37°C. Inhibition studies included adding 100 µM quinidine, 100 µM coumarin, 100 µM of quinidine and coumarin, with 60 µM (S)-nicotine.

Incubation of nicotine in yeast expressing CYP2D6. Incubation conditions using CYP2D6 expressed in yeast supported by cumene hydroperoxide (CuOOH), were essentially those of Zanger et al., (1988) and Wu (1993). Basically, CuOOH (80% in cumerol, Sigma) was first diluted to a concentration of 40 mM in 50% methanol in H_20 (v/v) and then to 375 µM in 0.3 M potassium phosphate buffer, pH 7.4. 200 µl of this solution was added to 100 µl nicotine (100 µM final) and 20 µl of rat liver cytosol, in a final volume of 1 ml (final CuOOH concentration of 75 µM). The incubation was initiated by the addition of 200 µl of yeast protein (0.3 mg/ml final) and was for 20 min. at room temperature. Incubations were carried out in a shaking water bath, at 37°C, for 120 min. All reactions were stopped with the addition of 100 µl 20% Na_2CO_3 (pH 11.4).

Results

Dextromethorphan to dextrorphan metabolism in human liver microsomes. The kinetics of dextrorphan formation were determined using a non-linear least squares algorithm in which the data were weighted by the reciprocal of the rate of metabolism and were fit by one or two site Michaelis-Menten kinetic models. The L11 liver sample displays a high affinity enzyme kinetics, while the L3 liver sample displays both high and low affinity enzyme kinetics. Low affinity enzyme sites were observed in 2 out of the 11 livers, while the remaining 9 livers had only a high affinity enzyme site with K_m and V_{max}

values (mean ± SD, n=9) of 5.79 ± 2.01 μM and 10.03 ± 6.53 nm les/mg protein/hr, respectively. Since the K_m value was approximately 5 μM, a 5 μM dose f dextr methorphan was used in incubations with 30 human liver microsomes. The rate of dextrorphan formation was used as a measure of CYP2D6 activity. PCR studies revealed two poor metabolizer genotypes (b/b) for CYP2D6 mediated reactions (L18, and L19), four heterozygote extensive metabolizers (wt/b: L26, L27, L61 and L63), with the remaining livers displaying the extensive metabolizer genotype (wt/wt).

Nicotine to cotinine kinetics

K_m and V_{max} values for nicotine to cotinine kinetics were calculated for all 30 human liver microsomes. Sample Michaelis-Menten curves for nicotine to cotinine kinetics are shown in Figure 4. These graphs show livers which display one site or multiple site enzyme kinetics. Figure 5 compares the respective K_m values across all 30 samples. These figures were segregated into male and female liver donors so that sex differences could be examined. The mean K_m value for all 30 livers is 66.6 ± 31.8 mM (mean ± SD). V_{max} results revealed marked inter-individual variations in cotinine formation (Figure 6). Four human livers appeared to have very high rates of cotinine formation. The V_{max} values between males and females was significantly different (p=0.07), but not when the four high female V_{max} values were removed (p=0.78), as determined by the students t-test. There is approximately a 30 fold difference in the V_{max} values of cotinine formation between the L32 and L60 liver microsome samples. The mean Vmax value for all 30 livers is 28.9 ± 28.9 nmoles/mg protein/hr (mean ± SD).

Correlation between CYP2D6 activity and cotinine formation

CYP2D6 as measured by dextromethorphan metabolism to dextrorphan was compared to nicotine to cotinine Vmax values across the 30 human livers. Specifically, the rate of dextrorphan formation was used as a measure of CYP2D6 activity. Results revealed no correlation (r=0.21, p=0.27), between CYP2D6 activity and cotinine formation.

Inhibition of cotinine formation

Quinidine, which is a specific inhibitor of the CYP2D6 enzyme, had some inhibitory effect on cotinine formation. Quinidine at 100 μM (1000 times greater than its K_i value for inhibiting dextromethorphan to dextrorphan metabolism by CYP2D6; Kerry et al., 1994) inhibited cotinine formation by approximately 20%. In the presence of 100 μM coumarin, cotinine formation was inhibited by over 80%, with little additional inhibition when quinidine was added in combination with coumarin.

Nicotine metabolism with yeast expressing CYP2D6

Nicotine incubations with yeast expressing CYP2D6 and yeast controls showed no difference in cotinine peak height ratios. The possibility of inactive CYP2D6-expressing yeast was investigated; p-methoxy-amphetamine, methamphetamine, and dextromethorphan, substrates for CYP2D6, were all oxidized by the CYP2D6-expressing yeast. Nicotine incubations in yeast were performed simultaneously with para-methoxyamphetamine incubations.

Discussion:

The metabolism of dextromethorphan and nicotine was studied using a bank of 30 human liver microsomes. Dextromethorphan metabolism to dextrorphan revealed typical Michaelis-Menten kinetics with some livers displaying a low affinity site. The apparent high affinity K_m value was 5.79 ± 2.01 (mean ± SD). Thus, 5 μM of dextromethorphan was incubated with 30 human liver microsomes to access CYP2D6 activity in each liver. Nicotine to cotinine metabolism was also investigated in the same 30 human livers. This required the development of a novel assay which is described above. Since the metabolism of nicotine to cotinine is a two step reaction, excess amounts of aldehyde oxidase was added to each incubation, such that the CYP oxidation step was rate determining. The formation of cotinine followed Michaelis-Menten kinetics with apparent K_m and V_{max} values (mean ± SD) of 66.7 ± 31.8 μM and 28.9 ± 28.9 nmoles/mg protein/hr, respectively.

Because of recent studies (Cholerton et al., 1994) which implicated a role for the CYP2D6 poor metabolizer phenotype of debrisoquine to the poor

metabolism of nicotine, CYP2D6's r le was addressed. Th first set f experiments were aimed at drawing a potential correlation between CYP2D6 activity and nicotine xidation t cotinine among 30 human liver microsomes. Dextromethorphan was used as the probe drug for CYP2D6 activity. Results revealed that liver microsomes which displayed low rates of dextrorphan formation was closely related to CYP2D6 genotyping studies. Dextrorphan rates of formation were plotted against nicotine to cotinine V_{max} values. No correlation was found between CYP2D6 activity and nicotine to cotinine metabolism (r=.21, n=30) suggesting that CYP2D6 is not a major enzyme involved in nicotine metabolism.

Correlation studies are not a conclusive form of evidence, thus supplementary studies were performed. Quinidine, which is a specific CYP2D6 inhibitor, had a small inhibitory effect on cotinine formation. At a very high concentration (100 μM) (K_i ~ 100 nM; Kerry et al., 1994) only a 20% inhibition was seen. In contrast coumarin, a specific CYP2A6 substrate, inhibited cotinine formation by over 80% at the same concentration. Stronger evidence excluding CYP2D6 from nicotine metabolism comes from the cDNA-expression work. Nicotine incubated with yeast microsomes expressing the human CYP2D6 enzyme revealed no cotinine formation greater than control yeast microsomes. These results are in disagreement with McCracken et al. (1992), but in agreement with Flammang et al., (1992).

The possibility of inactive yeast was investigated by incubating the yeast with known CYP2D6 substrates, such as dextromethorphan, p-methoxyamphetamine, and methylamphetamine. Results revealed that yeast expressing CYP2D6, and not control yeast, were able to oxidize these compounds to dextrorphan, para-hydroxyamphetamine, and para-hydroxymethylamphetamine, respectively. Further supporting evidence comes from Wu, 1993 who showed that nicotine did not inhibit dextromethorphan metabolism by CYP2D6 in human liver microsomes.

EXAMPLE 2

Role of CYP2A6 and CYP2B6 in Nicotine Metabolism

Since the CYP2D6 enzyme appears not to be involved in nicotine to cotinine metabolism, an investigation of the role of other cytochromes P450 was undertaken. In particular the importance of CYP2A6 in contributing to inter-individual differences in nicotine metabolism was accessed *in vitro*. CYP2A6, heterogously expressed in human lymphoblastoid cells, has one of the highest activities in the conversion of nicotine to cotinine, second only to CYP2B6 (McCracken et al, 1992).

The following materials and methods were utilized in the investigations outlined in Example 2:

MATERIALS AND METHODS

Human liver microsomes. The same 30 human liver samples were used in this study as were used in Example 1.

Drugs and chemicals. (S)-Nicotine, (S)-cotinine, NADPH, Tris-HCl, octanesulfonic acid, troleandomycin, orphenadrine, and ketamine were obtained from Sigma Chemical Co. (St.Louis, MO, USA). 7-methoxycoumarin, 7-methylcoumarin, and 7-ethoxycoumarin were purchased from Aldrich (St. Louis, MO, USA). Coumarin and ethyl acetate were obtained from Caledon (Georgetown, ON, Canada). Potassium phosphate was purchased from Mallinchrodt (Mississauga, ON, Canada). Antibodies were purchased from Gentest Corp. (Woburn, MA, USA).

Chemical inhibition studies. Extensive chemical inhibition studies consisted of incubating 100 µM (S)-nicotine with 150 µM concentrations of coumarin, orphenadrine, troleandomycin, and coumarin with orphenadrine in combination, with all 30 human liver microsomes, in duplicate. To maximize inhibition, and reduce the loss due to its own metabolism, a concentration of 150 µM was chosen for each specific inhibitor. From the kinetic studies this inhibitor concentration is approximately 2 times the mean K_m value for nicotine to cotinine metabolism. The nicotine concentration was set at 100 µM which was the concentration that was approaching the V_{max} for cotinine formation. This concentration was chosen to maximize the contributions of each cytochrome P450 involved in nicotine metabolism, since each enzyme

would likely have varying affinities for nicotine. Incubation conditions were similar to that mentioned in the previous example. Once coumarin was shown to inhibit nicotine metabolism, coumarin analogs were incubated with nicotine. High and low concentrations (10 and 100 μM) of coumarin, 7-methylcoumarin, 7-methoxycoumarin, and 7-ethoxycoumarin were incubated with 50 μM nicotine in K27 human liver microsomes.

Immunochemical inhibition studies. Immunoinhibition experiments consisted of incubating 0.5 mg/ml K27 liver microsomes with a CYP2A6 monoclonal antibody (MAB-2A6) and a CYP2B1 (anti-rat CYP2B1) polyclonal antibody. Antibodies and microsomes were preincubated on ice for 30 minutes followed by the addition of 100 μM nicotine, 1 mM NADPH, and 20 μl rat cytosol in 25 mM Tris-HCl buffer. Antibody concentrations were chosen based on immunoinhibition information provided by Gentest Corp. Figure 7 shows the potency and specificity of the MAB-2A6 and anti-rat CYP2B1 for their respective enzymes. Gentest Corp. stated that MAB-2A6 inhibited over 95% 2A6 activity, at 0.25 mg antibody/μg microsomal protein. They used coumarin hydroxylation as a measure of 2A6 activity. They also showed that anti-rat 2B1 cross-reacts with the human CYP2B6 enzyme to inhibit its activity.

Western Blot Analysis. Liver microsomal proteins (30 μg) were resolved on 10% SDS-PAGE gels, and transferred to nitrocellulose (120 volts, 18 hrs, at room temperature) by electroblotting (Western blotting) (Guengerich et al., 1982a). Blots were blocked for 1 hr at room temperature with 2% (wt/v) BSA dissolved in 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, and .05% Tween 20 (TBST). Incubations with primary and secondary antibodies were performed for 1 hr in TBST. The primary, and secondary antibodies consisted of the monoclonal CYP2A6 antibody (1/2000 dilution of 5 mg/ml stock; Gentest Corporation), and an anti-mouse IgG horseradish peroxidase conjugate (1/2000 dilution; Amersham Corporation, Arlington Heights, IL), respectively. After each incubation with primary and secondary antibodies, blots were washed three times with TBST for 10 minutes each. Blots were visualized using the chemiluminescent ECL reagent (Amersham Corporation). The densities of

the visualized bands were quantified using a MCID imaging system (Imaging Co.). After determining the linearity of detection of CYP2A6 bands (Figure 8), a concentration of 30 µg of microsomal protein was used for each liver for comparisons.

RESULTS

CYP2A6 in nicotine metabolism

Coumarin, a specific and selective CYP2A6 substrate significantly inhibited cotinine formation with a mean inhibition of 84 ± 11% (mean ± SD) (Figure 9 and 10). An apparent Ki value of ~2.0 mM (n=3) was determined using K27 human liver microsomes. A sample Dixon plot is shown in Figure 11. The competitive nature of this interaction was confirmed by performing Cornish-Bowden plots (Cornish-Bowden, 1974). Coumarin, along with three analogs of coumarin, were incubated with 50 µM nicotine in K27 liver microsomes. The inhibition results and the rank order of potency for coumarin, 7-methylcoumarin, 7-methoxycoumarin, and 7-ethoxycoumarin are shown in Figure 12. Of the four compounds coumarin had the strongest inhibitory effect on cotinine formation. Immunoinhibition experiments were carried out using a specific monoclonal antibody raised against 2A6. Results showed an over 50% inhibition of cotinine formation when 0.5 µg MAB-2A6/µg microsomes was incubated with 100 µM nicotine (Figure 13). Immunoreactive CYP2A6 was measured in each of the 30 human liver microsomes The densities of each band were used to compare the relative amounts of CYP2A6 between livers (Figure 14). Band densities were standardized by dividing them by the 30 µg L64 band density of their respective blots. This was done so that that individual band densities can be compared between blots. Western blot analysis was repeated at 3 and 10 µg amounts for livers that were outside the linear range, as determined by the L64 standard curves. A summary table of the values used in CYP2A6 and nicotine correlation studies is presented in Table 1. Using the band densities obtained from the Western blots, a strong correlation ($r=0.90$, $n=30$, $p<0.001$) was seen

between CYP2A6 levels and V_{max} values of cotinine formation (Figure 15). This r value decreases to 0.60 when the four high V_{max} livers were removed from the correlation. A stronger correlation was seen when CYP2A6 immunoactivity was plotted against the amount of cotinine inhibited in the presence of 150 µM concentrations of coumarin (r=0.94, n=30, p<0.001) (Figure 16). This r value decreases to 0.64 when the four high Vmax livers were removed. The V_{max}/K_m values which are listed in Table 1 provide an excellent measure for the efficiency of individual livers for metabolizing nicotine to cotinine. The higher the value the more efficient the liver. These V_{max}/K_m values were plotted against CYP2A6 immunoactivity which resulted in a strong correlation (r=0.94, n=30, p<0.001) (Figure 17). This correlation remained strong even when the four high V_{max} livers were removed (r=0.84).

CYP2B6 in nicotine metabolism.

Orphenadrine, which is a CYP2B6 inhibitor, had some slight inhibition which was approximately 20 ± 16% (mean ± SD) (Figures 18 and 19). When antibodies raised against the rat CYP2B1 were included, a 30 % inhibition of cotinine formation was seen in the K27 human liver microsomes (Figure 20). Coumarin and orphenadrine were also used in combination and had an overall 92 ± 11% (mean ± SD) inhibition of cotinine formation (Figure 21).

CYP3A4 in nicotine metabolism

Troleandomycin, a specific CYP3A inhibitor, did not show any overall inhibition of cotinine formation. The mean inhibition was 3% of control cotinine formation, with a standard deviation of 11% (Figure 22).

Discussion:

Since the metabolism of nicotine to cotinine by CYP2D6 was concluded to be of minor importance the role of other cytochromes P450 was investigated. In particular, the importance of CYP2A6 and CYP2B6 was addressed since both these enzymes are variably expressed in humans, and have been shown to contain some nicotine oxidase activity (Flammang et al., 1992; McCracken et al., 1992).

In the chemical inhibition studies cotinine pr duction was significantly inhibited after the additi n of coumarin, a specific CYP2A6 substrate (Pearce et al., 1992; Yamano et al., 1990; Waxman et al., 1985). Coumarin was incubated in the presence of nicotine, across the 30 human livers. Results indicated a universal inhibition of cotinine formation. Nicotine incubations in the presence of coumarin alone inhibited cotinine formation by over 80%, and when orphenadrine was added this value increased to 91%. In particular, when coumarin and orphenadrine were used in combination, 23 of the 30 livers showed a greater than 90% inhibition of cotinine formation. Coumarin inhibition of nicotine metabolism was found to be competitive and quite potent with a Ki of ~2.0 mM. Figure 12 summarizes the effect of coumarin along with three analogs in inhibiting cotinine formation. The rank order of potency was coumarin > 7-methoxycoumarin > 7-methylcoumarin > 7-ethoxycoumarin. It is interesting to note that 7-ethoxycoumarin had a lesser effect of inhibiting nicotine metabolism than coumarin, since 7-ethoxycoumarin is a well known substrate for many human cytochrome P450 enzymes (i.e. CYPs 1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2E1, and 3A4) (Waxman et al., 1991). This suggests that nicotine and coumarin metabolisms are closely related. Immunochemical inhibition studies revealed that a monoclonal antibody raised against the human CYP2A6 inhibited cotinine formation by 60%. CYP2A6 immunoactivity was also quite variable with a greater than 300 fold difference between L27 and L60 human livers. It is interesting to note that no detectable amount of this enzyme was found in the L32 liver sample, under these experimental conditions. Perhaps this individual carries variant mutant alleles for the CYP2A6 polymorphism. Western blot analysis revealed that nicotine metabolism was highly correlated to CYP2A6 levels. Nicotine to cotinine Vmax values correlated with CYP2A6 levels ($r=0.90$, $p<0.001$) across the 30 human livers. Using the coumarin inhibition results, which can be used as a measure of relative CYP2A6 activity, an even stronger correlation was seen with immunoreactive CYP2A6 ($r=0.94$, $p<0.001$). Also, CYP2A6 levels strongly correlated with V_{max}/K_m values ($r=0.94$, $n=30$, $p<0.001$).

From the information btained in example 1 it was shown that nicotine metabolism by the 30 human liver microsomes displayed large inter-individual variations. In particular, a 30 fold variati n was seen betwsen the lowest and highest metabolic rates. It is interesting to note that the four livers with exceptionally high cotinine formation were all females. *In vivo* studies, however, show that nicotine metabolism is more rapid in men than women (Beckett et al., 1971; Benowitz et al., 1984). There was no correlation with respect to cotinine formation and age. The differences in cotinine formation may be explained by variable expression of the CYP2A6 enzyme. The same four individuals who had exceptionally high nicotine oxidase activity also had large amounts of the CYP2A6 enzyme. One possible explanation is that the four livers showing high rates of nicotine metabolism were exposed to environmental inducers (i.e. phenobarbital) which would increase levels of CYP2A6. In summary this study indicates that CYP2A6 is very important in the human liver metabolism of nicotine.

With respect to CYP2B6, previous literature has shown that it is not constitutively expressed in human livers, and is likely induced by exposure to phenobarbital. One particular study has shown that detectable levels of this enzyme, as measured by Western blots, only occurred in 12 out of 50 livers (Mimura et al., 1993). In the present study orphenadrine and anti-rat 2B1 were used to investigate the importance of CYP2B6 in nicotine metabolism. Orphenadrine is an anti-parkinsonian agent which has been shown to form an inhibitory intermediate complex, in hepatic microsomes, only in phenobarbital induced microsomes (Reidy et al., 1989). Using 150 µM orphenadrine, in the chemical inhibition studies, resulted in an overall net inhibition of 20 ± 16% (mean ± SD). Antibodies raised against rat 2B1 has been shown to have affinity for the human CYP2B6 enzyme by Gentest Corp. They used similar concentrations of antibody to inhibit specific 2B6 mediated reactions. Previous cDNA work have shown that this enzyme has the highest known rate of nicotine oxidation (Flammang et al., 1992; McCracken et al., 1992). Therefore in certain individuals, who are exposed to environmental

inducers such as phenobarbital, CYP2B6 may play an important role in nicotine metabolism.

Trolandomycin, a substrate for several CYP3A enzymes, was used to study CYP3As role in nicotine metabolism. It was important to answer the CYP3A question since it is the most abundant CYP found in human liver (Shimada et al., 1994). CYP3A expression is also induced by exposure to phenobarbital, hence it is potentially a great source of variability in nicotine metabolism. Thus, the use of troleadomycin in the chemical inhibition studies behaved as a negative control. The results agree with previous studies that showed no involvement of the CYP3A subfamily in nicotine metabolism.

CYP2A6 has been shown to play an important role in nicotine metabolism, thus variations in CYP2A6 expression may be directly responsible for the high inter-individual variation seen in cotinine formation. Genetic variation in CYP2A6, and variable CYP2B6 expression may contribute to the 3-fold variation observed in nicotine metabolism in human subjects (Benowitz et al., 1982). Exposure to phenobarbital has been shown to have an inductive effect on nicotine metabolism by primarily increasing the expression of cytochrome P450 enzymes (Nakayama et al., 1982; Hibberd et al., 1985; Foth et al., 1990; Seaton et al., 1991; Seaton et al., 1993). Rat perfused livers, pretreated with phenobarbital, showed a 14-fold increase in nicotine elimination compared to saline treated controls (Rudell et al., 1987). Human hepatocyte studies, in which individuals were pretreated with phenobarbital, showed higher than normal nicotine oxidation rates (Williams et al., 1990). One particular study showed that the primate CYP2A mediated activity increases with exposure to phenobarbital (Pearce et al., 1992). The CYP2A and CYP2B gene subfamilies are closely linked on chromosome 19. Thus common factors may influence the expression of genes within these subfamilies in the human liver (Miles et al., 1989, 1990; Forrester et al., 1992). This implies that, along with CYP2B6, human CYP2A6 expression may be affected by exposure to phenobarbital. This, in turn, could affect the overall metabolism of nicotine from the body. Several studies have indicated that smokers adjust their smoking behavior to try and regulate or maintain nicotine blood levels

(McMorrow et al., 1983; Russel et al., 1987). Therefore rapid metabolizers of nicotine may smoke more cigarettes to maintain nicotine levels, and hence are exposed to more potentially toxic compounds. This can occur if individuals are exposed to phenobarbital, which has been shown to increase CYPs 2A6 and 2B6 mediated reactions. Conversely, slower metabolizers of nicotine may smoke less cigarettes so that toxic doses of nicotine are not achieved, or else they might be at a higher risk for nicotine-related adverse effects. This might occur in individuals who contain the variant/inactive forms of CYP2A6.

These studies have confirmed that CYP2A6 is important in nicotine metabolism, and that nicotine metabolism is quite variable among individual human liver microsomes. This variability may be a consequence of previous drug use, and in the case of CYP2A6 the presence of variant CYP2A6 alleles.

Example 3

A study has been designed to determine (i) if coumarin metabolic pattern (recovery and ratios of free, 7-OH and conjugated 7-OH coumarin) reflects CYP2A6 genotype, independent of current smoking status, and nicotine (NIC) metabolism determined from smokers' blood and urine samples; (ii) if acute smoking affects coumarin metabolism in smokers; and (iii) if there are differences between male and females in the prediction of NIC metabolism from coumarin metabolism.

Equal numbers of medication free, healthy males and females of any racial background currently tobacco dependent (n = 30 of each sex) and non-smoking (n = 30 of each sex) will be genotyped (CYP2A6) and will have a Coumarin Test on 2 separate days, once between 7:00 and 9:00 a.m. and once between 2:00 and 4:00 p.m. Smokers will be required to have the morning test after abstaining from smoking for at least 8 h (for example 8 a.m. before the first cigarette) and the afternoon test on a normal smoking day. Urine and plasma samples, which can be analyzed for NIC and cotinine (COT) (free and conjugated), will be taken prior to each Coumarin Test, along with breath carbon monoxide to determine smoke exposure. Coumarin and free and total

7-OH c umarin will be analyzed by HPLC using U.V. detection with 4-OH coumarin as an internal standard after Rauti et al.

Example 4

C-2 Acute Effects of CYP2A6 Inhibition on NIC Disposition and Smoking

Benowitz has used a 30-minute deuterium-labelled NIC-d2 infusion (with COT-d4) to study the kinetics and fractional clearance of NIC in smokers and non-smokers. The kinetics of the deuterated NIC are very similar to unlabelled NIC. The advantage of the non-radioactive label is that the NIC-d2 and resulting COT-d2 can be used as quantitative measures of NIC metabolism in smokers while smoking. This approach will be used to obtain a quantitative estimate of NIC to COT conversion by giving a dose of NIC-d2 sufficient to detect in the urine, taking advantage of the fact that NIC and COT are found in much higher concentrations in urine than in plasma. In preliminary studies smokers infused with 2µg/kg/min x 30 minutes were found to have urine concentrations of NIC and COT that were >80 and >600ng/ml, respectively. Therefore it is estimated that as little as 0.2 to 0.8 mg of NIC-d2 will result in quantifiable NIC-d2/COT-d2 in the urine. This would correspond to a 6 minute infusion of the 0.5 µg and 2.0 µg/kg/30 min infusions of Benowitz in non-smokers and smokers. Ratios of NIC-d2/COT-d2 in urine collected over 8 h, "Nicotine-d2 Test", will provide a direct estimate of NIC to COT conversion. This procedure will be piloted to establish dose, dose rate and analytic sensitivity. Plasma and urinary NIC, COT and trans-3'-hydroxycotinine and their glucuronides will be measured using a existing GC assay as modified in Dr. Jacob's lab (1988 protocol). Conjugates will be determined after alkaline hydrolysis (NIC and COT) or hydrolysis with β-glucuronidase (3'-hydroxycotinine). The quantitation limit is 1 ng/ml NIC and 10 mg/ml COT, with detection 50% lower. Coefficients of variation range from 1.1 to 7.8% for NIC (1-100 ng/ml). NIC-d2, COT-d2 will be determined by GC-MS (111).

The results of the experiments described in Example 2 indicate that CYP2A6 is the primary contributor to NIC disposition, with CYP2B6 important

in a few individuals (= 16%). Coumarin is a potent inhibitor of NIC metabolism to COT *ex vivo* in human liver microsomes (Ki = 1.5μM), and orphenadrine is a potent inhibitor of human CYP2B6 (Ki = 3.8 μM), with an estimated half-life f about 14 h. Most smokers' NIC metabolism can be inhibited by coumarin, the remainder by an appropriate combination of coumarin and orphenadrine; and such inhibition will reduce smoking behaviour.

A study has been designed to determine the extent to which combinations of inhibitors of CYP2A6 and CYP2B6 will modify NIC metabolism *in vivo* and smoking behaviour in a social controlled setting.

CYP2A6 genotyped current tobacco dependent (DSM-IV) individuals (n = 6 wt/wt, n = 6 wt/mut) will have CYP2A6 activity assessed on six occasions by a "Coumarin Test" along with measurement of plasma NIC and COT. After familiarization with the inviting social setting study site, subjects will abstain from smoking until they arrive on each study day, when they will provide a baseline urine (NIC/COT), a plasma sample (NIC/COT), and a breath CO. They will receive, on separate days, all combinations of 3 coumarin conditions (placebo, 50 mg b.i.d. or 100 mg b.i.d.) with 2 orphenadrine conditions (placebo or 200 mg); the two combinations of two active drugs will occur on days 5 and 6, after tolerance to the individual components has been verified on days 1-4 in a random, counterbalanced order. A great deal of work has been done to establish the relationship of number of cigarettes, NIC intake, smoke exposure and NIC and COT plasma (blood) concentrations and urinary excretion. The best correlations are obtained between blood NIC (4:00 p.m., 0.79), CO Hbg (0.67), urinary COT 24 h (0.62), blood COT (4:00 p.m., 0.53). COT, because of its longer half-life is less critically affected by sampling time and can be used to estimate daily NIC intake. 30 minutes after study drug, a tracer dose of Nicotine-d2 Test 1 will be given. Subjects will then collect their urine for 3 h ("Coumarin Test") and 4 h separately for determination of NIC-d23/COT-d2 ratio (7 h) and coumarin and total and free 7-OH coumarin. Additional blood samples will be collected 0.5 (coumarin/7-OH-coumarin), 3 and 7 h (NIC/COT)

after the pulse tracer dose commences. Breath CO will be determined at the same times. During this period subjects will be permitted to smoke their usual brand ad lib, drink caffeinated beverages, play games, watch videos, etc. The number f cigarettes used and residual butt weight recorded.

Example 5

C-3 Smoking Reduction by Inhibition of CYP2A6

Inhibition of metabolism of NIC to COT will allow smokers to maintain plasma NIC with less smoke exposure and reduce the secondary reinforcement of smoking behaviour as a component of eventual smoking cessation. Because NIC is the addictive agent in tobacco dependence and smokers regulate their brain NIC within a fairly narrow individual concentration band, selective inhibition of NIC conversion to COT should result in a decrease in smoke exposure (i.e. "smoking").Some individuals may require different combinations of CYP2A6 and CYP2B6 inhibitors to achieve sufficient modification of NIC metabolism..

A preliminary study has been designed to confirm the efficacy and safety of CYP2A6 inhibitors and the need for CYP2B6 inhibition in the reduction of smoking exposure and as an aid in smoking cessation.

Male or female DSM-IV dependent tobacco users who want to stop smoking, who do not want NIC substitution treatment, have made at least three serious unsuccessful attempts to stop, have no medical contraindications to participation, and have CYP2A6 wt/wt or wt/mut genotype will be eligible to participate. Prior to the trial, susceptibility to CYP2A6 activity inhibition will be assessed by stable-labelled "Nicotine Test" after coumarin 50 or 100 mg b.i.d. (Human Study C-1), to assess the change in the individual urinary NIC-d2/COT-d2 ratio. Based on the results of the study each subject will be assigned to a CYP2A6 inhibition "responders" or "poor responders" group. Therefore the study will be a comparison of placebo (n = 30) vs. coumarin (n = 30) in CYP2A6 high inhibition responders and of coumarin (n = 7) vs. coumarin + orphenadrine (n = 8) in CYP2A6 poor responders for 2 weeks. The coumarin doses may be 100 mg b.i.d. and the orphenadrine 100 mg p.o. daily.

Tobacco sm king behaviour will be monit red by daily smoking diary cards, twice weekly home carbon monoxide breath sample collected in remote CO exposure collection bags (mid-afternoon between 2:00 and 6:00 p.m.), twice weekly salivary COT. Subjects will be given instructions with respect to the purpose of treatment, plus limited supportive counselling, plus structured self-help advice. Patients will be seen weekly between 2:00 and 6:00 p.m. each day at which time plasma NIC/COT, breat. CO will be done.

The primary dependent variables are measures of smoke exposure (diaries and CO measurements), averaged over the 2-week study; such a mean is sensitive to both downward and leftward shifts of the consumption-over-time curve. These variables will be analyzed as a function of treatment within high and poor responding groups separately. For two-group comparisons, n=30 per group has approximately 97% power to detect a difference of 1 S.D. between their means.

Example 6

C-4 CYP2A6 Inhibition in Smoking Reduction and Cessation

Based upon studies such as outlined in Example 5, a double-blind trial to confirm the efficacy of CYP2A6 inhibition on smoking reduction and cessation will be carried out. The drug choice and doses will be determined by the studies as outlined in Example 5. A positive treatment control (e.g. NIC patch) will be tested.

Patients identical to those participating in the previous study (Human Study C-3) will enter a double-blind placebo controlled randomized trial of smoking reduction and cessation to be achieved and maintained over 12 weeks comparing coumarin and placebo (n=60) per group. The assessment and procedures will be similar tot he study as outlined in Example 5. However, those actually receiving the CYP2A6 inhibitor will receive active drug for a 2-week period followed by a 2-week placebo period. This 4 week on/off cycle will be repeated 3 times with a goal of cessation at the end of 12 weeks. CYP2A6 inhibition should decrease smoke exposure by decreasing the number of cigarettes or by altering smoking behaviour. Subjects will, at the end of each "active" drug/placebo 2-week phase, be told to maintain their

lower sm king behavi ur for 2 weeks. This 2-week period is one of behavioral change. The inhibitor-placebo cycle will be repeated. Subjects will be seen weekly at which time their self-report sm king logs will be reviewed along with their progress (minimal adjunctive care). After the trial, subjects will be contacted at 3, 6 and 12 months and provide at least a salivary COT to determine maintenance of quit rates. Breath CO, salivary, plasma NIC/COT will be determined. Established criteria for cessation will be used.

Having illustrated and described the principles of the invention in a preferred embodiment, it should be appreciated to those skilled in the art that the invention can be modified in arrangement and detail without departure from such principles. We claim all modifications coming within the scope of the following claims.

All publications, patents and patent applications referred to herein are incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

Below full citations are set out for the references referred to in the specification.

TABLE 1

Summary table of values used in CYP2A6 correlation studies.

Liver #	Age	Sex	ζ CYP2A6 immunoactivity	ξ Coumarin inhibition of cotinine formation (pmoles/mg protein/hr)	Km (μM)	Vmax (pmoles/mg protein/hr	Vmax/Km (μL/mg protein/hr)
K20	50	F	1.336	10.264	78	28.5	0.365
K27	55	F	2.080	9.368	64	28.4	0.444
L18	53	F	2.150	8.603	22	8.2	0.373
L20	47	F	0.745	11.829	52	16.8	0.323
L24	3–4	F	5.100	42.096	50	68.0	1.360
L31	36	F	3.811	15.048	38	22.4	0.589
L32	8	F	0.000	1.243	106	4.2	0.040
L36	30	F	5.610	50.744	60	96.1	1.602
L38	16	F	4.620	49.045	56	98.3	1.755
L39	19	F	1.948	19.643	52	25.2	0.485
L44	36	F	2.017	16.653	73	34.2	0.468
L45	5	F	1.498	16.712	68	26.8	0.394
L60	16	F	10.520	99.021	55	120.0	2.182
L61	46–47	F	0.042	5.096	131	9.1	0.070
L62	55	F	0.176	4.595	69	8.5	0.123
L63	42	F	0.752	6.723	66	16.5	0.250
Mean +/- SD			2.650 2.752	22.918 25.592	65.900 25.356	38.200 36.526	0.676 0.661

TABLE 1 (CONT'D)

L19	6--7	M	1.678	14.891	46	23.6	0.513
L25	13	M	0.646	5.947	60	10.4	0.173
L26	31	M	1.667	10.264	68	15.8	0.232
L27	17	M	0.034	2.333	162	7.5	0.046
L29	9	M	2.407	10.024	45	18.2	0.404
L30	23	M	0.240	3.912	51	6.5	0.127
L33	2	M	0.239	2.443	123	8.4	0.069
L34	28	M	3.203	16.053	30	22.2	0.740
L40	49	M	2.687	19.629	60	34.8	0.580
L41	4	M	0.555	12.845	91	24.8	0.273
L43	42	M	1.799	22.536	42	34.8	0.829
L47	43	M	1.028	6.741	21	7.8	0.373
Mean +/- SD			1.349 1.052	10.635 6.700	66.583 40.592	17.900 10.273	0.363 0.258
L23	8	Unknown	0.989	11.053	56	17.3	0.309
L64	4	Unknown	0.771	20.347	104	22.6	0.217
Mean +/- SD			0.880 0.154	15.700 6.571	80.000 33.941	19.950 3.748	0.263 0.065

ζ Values were calculated from band densities obtained from Western blots.
ξ Amount of cotinine formation inhibited by the addition of 150 mM coumarin.

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WE CLAIM:

1. A method f regulating nicotine metabolism in an individual comprising selectively inhibiting CYP2A6.

2. A method as claimed in claim 1 wherein CYP2A6 is sclectively inhibited using one or more of the following (i) substances which inhibit CYP2A6 activity; or (ii) substances which inhibit transcription and/or translation of the gene encoding CYP2A6.

3. A method of screening for a substance that regulates nicotine metabolism to cotinine in an individual comprising assaying for a substance which (i) selectively inhibits CYP2A6 activity, or (ii) selectively inhibits transcription and/or translation of the gene encoding CYP2A6.

4. A pharmaceutical composition for use in treating a condition requiring regulation of nicotine metabolism to cotinine comprising an effective amount of a compound which selectively inhibits CYP2A6, and a pharmaceutically acceptable carrier, diluent, or excipient.

5. A method for treating a condition requiring regulation of nicotine metabolism to cotinine in an individual comprising administering to the subject an effective amount of a compound which selectively inhibits CYP2A6.

6. The method as claimed in claim 5 whercin the condition is dependent or non-dcpendent tobacco use.

7. A method for enhancing inhibition of nicotine metabolism by a CYP2A6 inhibitor in an individual comprising administering to the individual an effective amount of a substance which selectively inhibits CYP2A6, and an effective amount of an inhibitor of CFY2B6.

8. A pharmaceutical compositi n for use in treating a condition requiring regulation of nic tine metabolism to cotinine c mprising an effective am unt of a substance which selectively inhibits CYP2A6, an effective amount of an inhibit r of CPY2B6, and/ r a pharmaceutically acceptable carrier, diluent, or excipient.

9. A method for treating a condition requiring regulation of nicotine metabolism to cotinine in an individual comprising administering to the individual an effective amount of a substance which selectively inhibits CYP2A6, and an effective amount of an inhibitor of CPY2B6.

ABSTRACT OF THE DISCLOSURE

A method f regulating nicotine metabolism in an individual comprising selectively inhibiting CYP2A6.

5

FIGURE 1

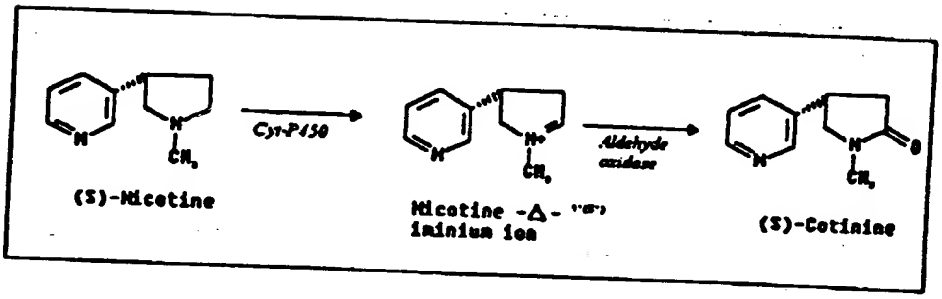
Cyt-P450
Aldehyde oxidase
(S)-Nicotine
Nicotine -Δ-
iminium ion
(S)-Cotinine

FIGURE 2A

```
LOCUS       HSU22027     7215 bp    DNA             PRI       22-OCT-1995
DEFINITION  Human cytochrome P450 (CYP2A6V2) gene, complete cds.
ACCESSION   U22027
NID         g1008461
KEYWORDS    .
SOURCE      human.
  ORGANISM  Homo sapiens
            Eukaryotae; mitochondrial eukaryotes; Metazoa; Chordata;
            Vertebrata; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 7215)
  AUTHORS   Fernandez-Salguero,P., Hoffman,S.M., Cholerton,S., Mohrenweiser,H.,
            Raunio,H., Rautio,A., Pelkonen,O., Huang,J.D., Evans,W.E.,
            Idle,J.R. et.al.
  TITLE     A genetic polymorphism in coumarin 7-hydroxylation: sequence of the
            human CYP2A genes and identification of variant CYP2A6 alleles
  JOURNAL   Am. J. Hum. Genet. 57 (3), 651-660 (1995)
  MEDLINE   95397851
REFERENCE   2  (bases 1 to 7215)
  AUTHORS   Fernandez-Salguero,P.
  TITLE     Direct Submission
  JOURNAL   Submitted (01-MAR-1995) Pedro Fernandez-Salguero, National
            Institutes of Health, 9000 Rockville Pike, Bethesda, MD 20894, USA
FEATURES             Location/Qualifiers
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     5'UTR           782..790
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                     4257..4398,4873..5060,5577..5718,6308..6489)
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                     /codon_start=1
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                     PMLGSVLRDLRFFSNPRDFNPQHFLGEKGQFKKRDAFVPFSIGKRNCFGEGLARMELF
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                     /number=1
     exon            1237..1399
                     /gene=CYP2A6V2"
                     /number=2
     exon            2115..2264
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                     /number=3
     exon            2499..2659
                     /gene=CYP2A6V2"
                     /number=4
     exon            3207..3383
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                     /number=5
     exon            4257..4398
                     /gene=CYP2A6V2"
                     /number=6
     exon            4873..5060
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                     /number=7
     exon            5577..5718
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FIGURE 2A CONT'D

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                     /number=8
          exon       6308..6489
                     /gene=CYP2A6V2"
                     /number=9
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       61 gaggttctat ggcagccatc ctggcctcac tctgaggttc caatgaggat tctgggcatc
      121 aagagacagc tctgggcaaa gctaaatcaa gtcagcccct ggacccagtg ctgggctgct
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     3121 ccttgctatg aaacaaatcc cattcccatc agctcctgcc ccgtgacagc tgtccttccc
     3181 ttcccatcct ctctctgcaa ccccagctct atgagatgtt ctcttcggtg atgaaacacc
     3241 tgccaggacc gcagcaacag gcctttcagt tgctgcaagg gctggaggac ttcatagcca
     3301 agaaggtgga gcacaaccag cgcacgctgg atcccaattc cccacgggac ttcattgact
     3361 cctttctcat ccgcatgcag gaggtacacc ccagcagcca ctgcggggag atgcaaagcc
```

FIGURE 2A CONT'D

```
3421 aggcagaggg aaatcagtct gggagtgggg caggcagatg acacaggccc attcaaaatta
3481 accctcatca taataatcct cacaattggc tgggtgccgt ggctaacagc ctgtaatccc
3541 agcactttgg gaggccgagg caggtggatc acctgaggtc aggagttcga gaccagcctg
3601 gccaacatgg tcaaaccccg tctctactaa aaatccaaaa attagttggg cgtggtggcg
3661 cgaagyyyyy cagaggttgc aatgagccaa gatcacggca ttgcactcca gtctgggtga
3721 cagaatgagg cctgtgtca aaaaaaatta atcacttgtt taaaaaagtaa gtgagcctgc
3781 atggtcatgc gcatgtgcag ctccagctac tcaggaggct gaggctggag gattgcttga
3841 gctcaggagt tggcgtccgg cctgtgcaac ttagcaagac caagtcagta taagaaaaaa
3901 aaaaaacaaa aaaaaagctg acagctaagt tgataattga cggacagatg gtcagcaagg
3961 taacgaaggt gagaaggaag agcattgggg gcaacgccag gagtcagggc aaggggctggt
4021 tcctagagcg agtctggtag gatctagggc cctctttctc caccctgcgg tcttgcccca
4081 aagagaggtc gagggtgctg ggattgcgct agactcgagt ctgtgtagat cttggggtcc
4141 cctcttgacc cccattggtc tgaacctaag agtggaagat ccatgggtg aacccctaga
4201 tggtgccctg aggtcaagca ggagtgaggt tgtcctaaag ccccctctcc cttcaggagg
4261 agaagaaccc caacacggag ttctacttga agaacctgat gatgagcacg ttgaacctct
4321 tcattgcagg caccgagacg gtcagcacca ccctgcacta tggcttctta ctgcttcatga
4381 agcacccaga ggtggaggct aaggctggag ggggacggaa gtggagggcc ccagacccte
4441 aaaatttccc ttcgactggt gcaatgtccc caactgtccc agatcccggg accctgagac
4501 gtgacttgct gtccagagac agggcaacat tcagctggta ggcatcagct gagtctcatt
4561 agatattaaa atattgaaaa tgtctgcact gattggtcag tcacttctgt cccaagccca
4621 ctgagtgccc actgcccgtt ccaccgggtc atcccctaag ttcctcccta tgcctcccct
4681 gtgattctgg cacaacctgg ttaacaggat cctactccaa caatgcgaat gggtgatgtc
4741 tgttctgtta tgaatgctct acttccgtct cataggcgga ggcatttcat ccaccccatt
4801 ttgcctatcc ggactatcat ttcctgctct gagaccccta gatacctaaa cacattcccc
4861 ctcctccccc agccaaggtc catgaggaga ttgacagagt gatcggcaag aaccggcagc
4921 ccaagtttga ggaccgggcc aagatgccct acatggagge agtgatccac gagatccaaa
4981 gatttggaga cgtgatcccc atgagtttgg cccgcagagt caaaaaggac accaagtttc
5041 gggatttctt cctccctaag gtgctatccg ccccaccccc ccagactacg gggactccag
5101 ccccctctcg tgtccccagc atcccaccca cattagaagc tttctagacc ctgtcccact
5161 ccctcaatca gtcaaaaaag acttccccaa ccaccacatc cgttccaccc ttccacttag
5221 acactcctga gtcctgcatc tctccagact ctttgtgtca cgagaatcaa acacatgttc
5281 ccaaacttcc tatcttaaga aacagaagcc ccctttccat tcggcctttt gtcataggga
5341 cagaaatctc aggtccccca aactcctgcc tagaaggaca tggacccccat gtctcccaaa
5401 cttcctgttt cagagatgtg aaccttctat ccccccaaggt cctccctcag aggtccccaa
5461 ttcccatgcc tgccacttcc cctcaccggg gcaccctagt tccccctcca gccccctgtgt
5521 actctcaaca atccccccaac ccgcctcatc acatacacct tcctcctccc tcccagggt
5581 tagaagtgtt ccctatgttg ggctccgtgc tgacagacct caggttcttc tccaaccccc
5641 gggacttcaa tccccagcac ttcctgggtg agaaggggca gtttaagaag cgtgatgctt
5701 ttgtgccctt ctccatcagt aagagaccac tgtttggtgc caggcttact actcacacca
5761 gcaggggcct cccttaccca gttcccctct ctgccgtgta gcctagtatt tccccagctt
5821 ggcaagttcc tgttagcaat ctaccgtcga gccaccaggt gatactccct taactaccaa
5881 gcacccagta cctgtgccca ggcaaaagga aaggaaacat catacccctt tcagaggcgg
5941 gggaaaacca aaggccagag agaatcagag atttatttcc ctagggtcac acaggagatt
6001 cttcagcatc cctaaaaagg agatgacggc acagcaggtc atatttggga gttcttatct
6061 gggggaaggg ggatcttaaa cctcccattg tggacacctg gcatcgatca accccatctt
6121 ttggtcatct tttgggtcac tcaaggaaac tgaggtcaag gagggtcaag aggctccctc
6181 ttaaagtctc tcagggccat atattccacc cttcctccct gggagagccg cagctggagg
6241 tcggtactgg ggcgaggctg cactgagagt gggcttcacc tccacccctc ccgcctctcc
6301 tcctcaggaa agcggaactg tttcggagaa ggcctggcca gaatggagct cttttcttc
6361 ttcaccaccg tcatgcagaa cttccgcctc aagtcctccc agtcacctaa ggacattgac
6421 gtgtccccca aacacgtggg ctttgccacg atcccacgaa actacaccat gagcttcctg
6481 ccccgctgag cgagggctgt gccggtgaag gtctggtggg cggggccagg gaaagggcag
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6601 aaggggcgtg gtggctagag ggaagagaag aaacagaagc ggctcagttc accttgataa
6661 ggtgcttccg agctgggatg agaggaagga aacccttaca ttatgctatg aagagtagta
6721 ataatagcag ctcttatttc ctgagcacgt accccgtgt caccttttgtt caaaaaccat
6781 tgcacgctca cctaatttgc cacaaaaccc ccttcgaagg ggcgttcatg cccatttttac
6841 acgtgacaaa actgaggctt agaaagttgt ctctgatgtc tcacaaaaca taagtgccca
6901 gaaaatctgc gaacacagat ctgtgcccat agccttctag acagattctt aaaaagcacc
6961 tattcctcac gcaaaacagt ttagtataga atcacatggc ctgaacatcc ctgtccgggg
7021 gagttcccca gagacctggg gggtggttgc cctgccttca ctgcacacat gcccacactc
7081 tcacctactc aacatgctgt gactacccgg gtgtaatctg tgcttgctac cagataaggc
7141 cactgtagcc cattcagagt cagcccaggg acacaacgag acatgactgg acatacaggg
7201 tcagtccatt aacaa
```

//

FIGURE 2B

```
LOCUS       HSP452B6     1415 bp    RNA             PRI       29-MAY-1992
DEFINITION  Human mRNA for cytochrome P-450IIB6.
ACCESSION   X13494
NID         g35206
KEYWORDS    cytochrome; cytochrome P450IIB6.
SOURCE      human.
  ORGANISM  Homo sapiens
            Eukaryotae; mitochondrial eukaryotes; Metazoa; Chordata;
            Vertebrata; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 1415)
  AUTHORS   Miles,J.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (10-NOV-1988) Miles J.S., Imperial Cancer Research Fund,
            Lab of Molecular Pharmacology and Drug Metabolism, Hugh Robson
            Building, George Square, Edinburgh, EH8 9XD
REFERENCE   2  (bases 1 to 1415)
  AUTHORS   Miles,J.S., McLaren,A.W. and Wolf,C.R.
  TITLE     Alternative splicing in the human cytochrome P450IIB6 gene
            generates a high level of aberrant messages
  JOURNAL   Nucleic Acids Res. 17 (20), 8241-8255 (1989)
  MEDLINE   90045947
COMMENT     The sequence is a compilation of genomic and cDNA clones. **map:
            chromosomal location=19q12-13.2;
            Data kindly reviewed (13-NOV-1989) by Miles J.S.
FEATURES             Location/Qualifiers
     source          1..1415
                     /organism="Homo sapiens"
     misc_feature    9..110
                     /note=exon 1, partial"
     misc_feature    111..273
                     /note=exon 2"
     misc_feature    274..423
                     /note=exon 3"
     misc_feature    424..584
                     /note=exon 4"
     misc_feature    585..761
                     /note=exon 5"
     misc_feature    762..903
                     /note=exon 6"
     misc_feature    904..1091
                     /note=exon 7"
     misc_feature    1092..1233
                     /note=exon 8"
     misc_feature    1234..1415
                     /note=exon 9, coding region"
BASE COUNT      341 a    430 c    328 g    316 t
ORIGIN
        1 gaattccgcc ctgcacccat gaccgcctcc caccagggcc ccgccctctg ccccttttgg
       61 gaaaccttct gcagatggat agaagaggcc tactcaaatc ctttctgagg ttccgagaga
      121 aatatgggga cgtcttcacg gtacacctgg gacccaggcc cgtggtcatg ctgtgtggag
      181 tagaggccat acgggaggcc cttgtggaca aggctgaggc cttctctggc cggggaaaaa
      241 tcgccatggt cgacccattc ttccggggat atggtgtgat ctttgccaat ggaaaccgct
      301 ggaagggtgct tcggcgattc tctgtgacca cratgaggga cttcgggatg ggaaagcgga
      361 gtgtggagga gcggattcag gaggaggctc agtgtctgat agaggagctt cggaaatcca
      421 agggggccct catggacccc accttcctct tccagtccat taccgccaac atcatctgct
      481 ccatcgtctt tggaaaacga ttccactacc aagatcaaga gttcctgaag atgctgaact
      541 tgttctacca gacttttttca ctcatcagct ctgtattcgg ccagctgttt gagctcttct
      601 ctggcttctt gaaatactct cctggggcac acaggcaagt ttacaaaaac ctgcaggaaa
      661 tcaatgctta cattggccac agtgtggaga agcaccgtga aaccctggac cccagcgccc
      721 ccaaggacct catcgacacc tacctgctcc acatggaaaa agagaaatcc aacgcacaca
      781 gtgaattcag ccaccagaac ctcaacctca acacgctctc gctcttcttt gctggcactg
      841 agaccaccag caccactctc cgctacggct tcctgctcat gctcaaatac cctcatgttg
      901 cagagagagt ctacagggag attgaacagg tgattggccc acatcgccct ccagagcttc
```

FIGURE 2B CONT'D

```
 961 atgaccgagc cagcatgcca tacacagagg cagtcatcta tgagattcag agatttttcg
1021 accttctccc catgggtgtg ccccacattg tcacccaaca caccagcttc cgagggtaca
1081 tcatccccaa ggacacagaa gtattttcta tcctgagcac tgcttctccat gacccacact
1141 actttgaaaa accagacgcc ttcaatcctg accactttct ggatgccaat ggggcactga
1201 aaaaqactga agcttttatc cgtttgtcct tagggaagcg gatttgtctt gggtaaggca
1261 tcgcccgagc ggaattgttc ctcttcttca ccaccatcc: ccagaacttc tccatggcca
1321 gccccgtggc cccagaagac atcgatctga caccccagga gtgtggtgtg ggcaaaatac
1381 ccccaacata ccagatccgc ttcctgcccc gctga
//
```

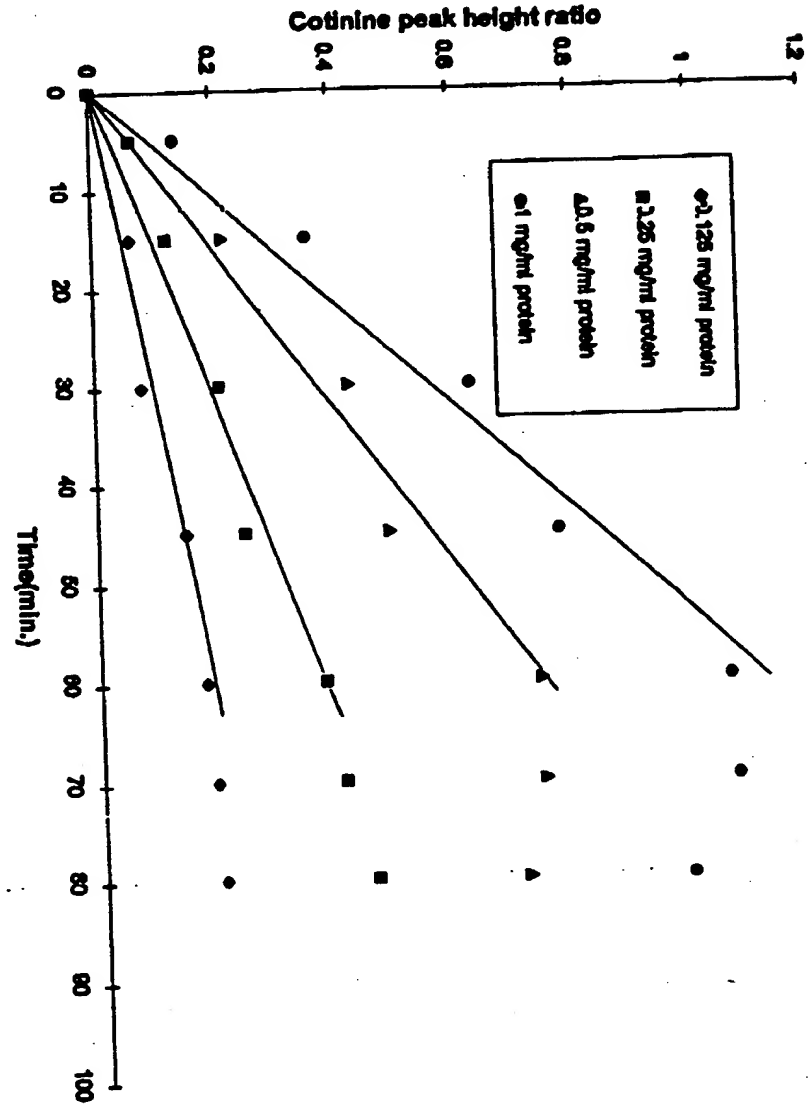
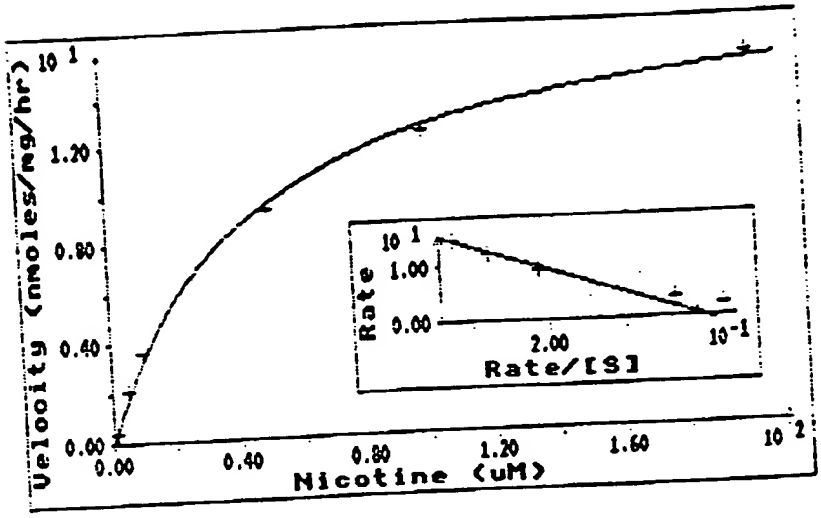


FIGURE 3

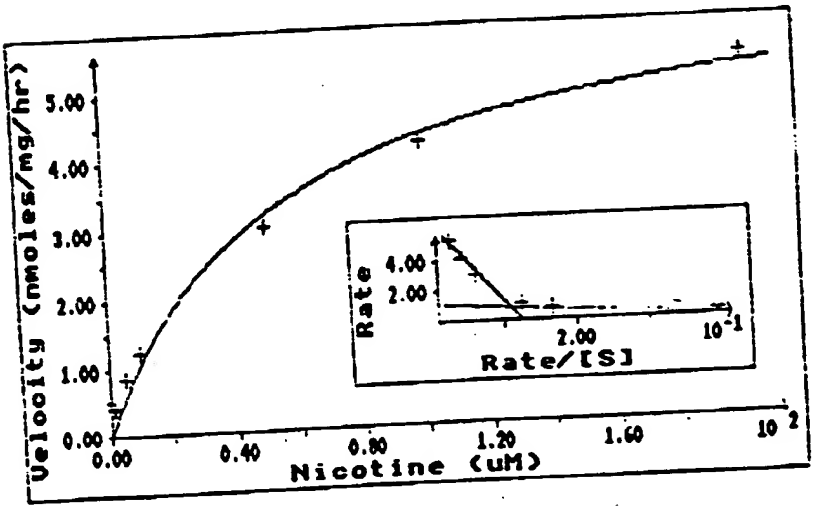
Protein-time curves of cotinine production from 100 μM nicotine in the presence of 20 μl rat cytosol by K20 human liver microsomes.

FIGURE 4

A


Velocity (nmoles/mg/hr)
10 1
1.20
0.80
0.40
0.00
0.00
0.40
0.80
1.20
1.60
10 2
Nicotine (uM)
Rate
10 1
1.00
0.00
2.00
10-1
Rate/[S]

B


Velocity (nmoles/mg/hr)
5.00
4.00
3.00
2.00
1.00
0.00
0.00
0.40
0.80
1.20
1.60
10 2
Nicotine (uM)
Rate
4.00
2.00
2.00
10-1
Rate/[S]

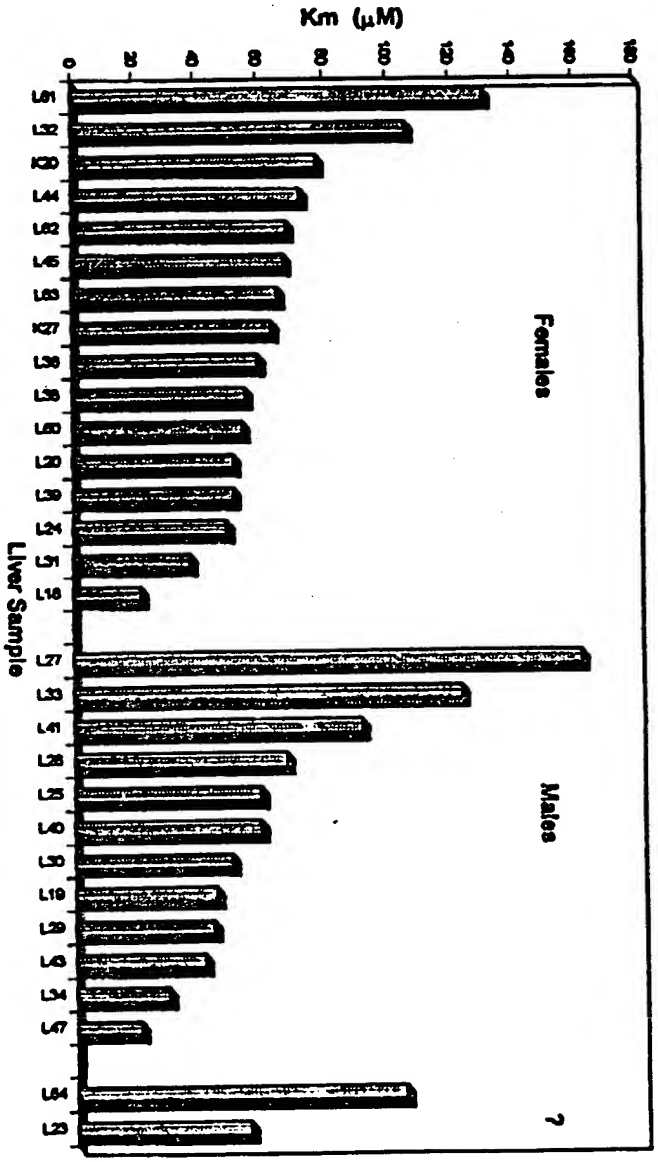


FIGURE 5

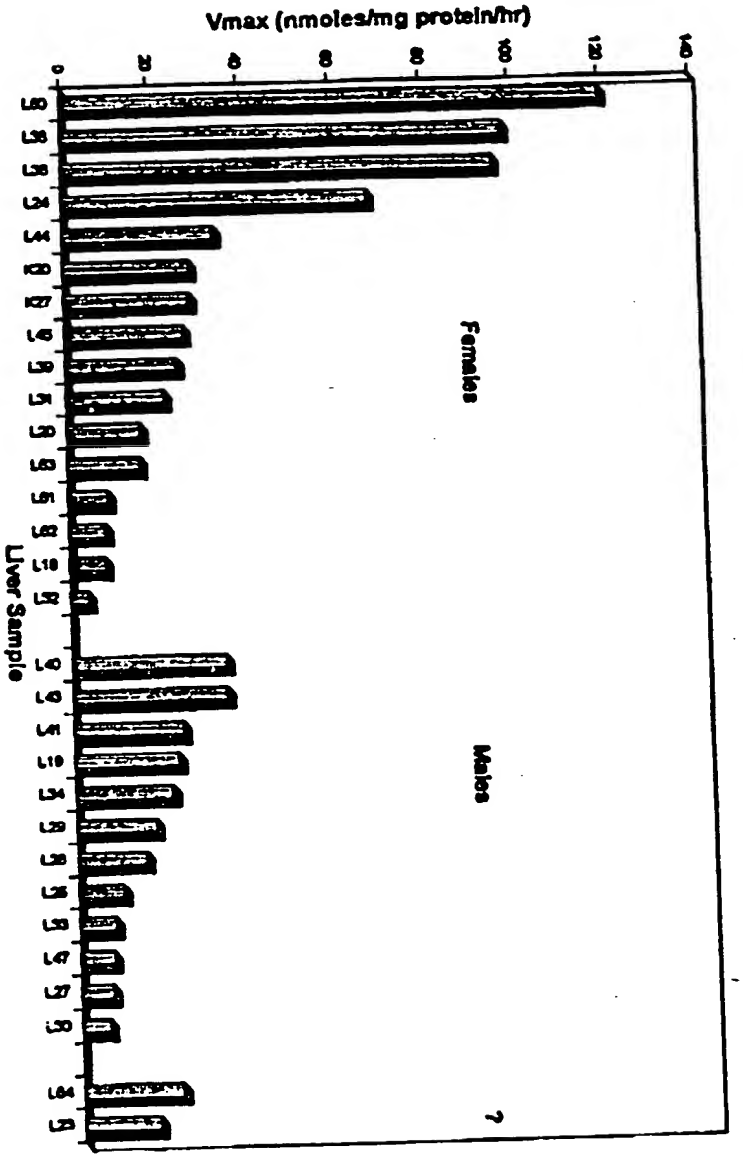
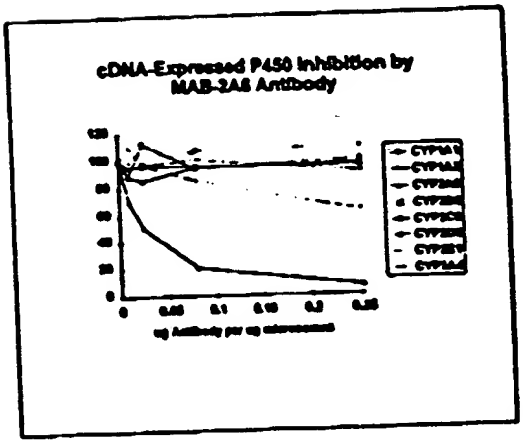

Vmax (nmoles/mg protein/hr)
0
2
4
6
8
10
12
14
Females
Males
Liver Sample

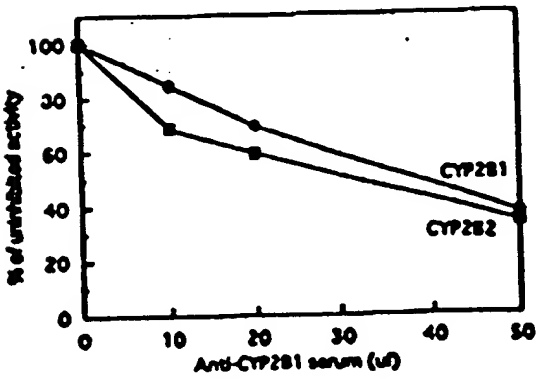
FIGURE 6

FIGURE 7

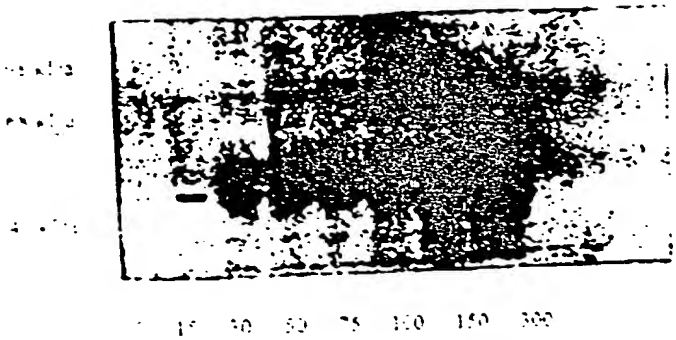
(A)

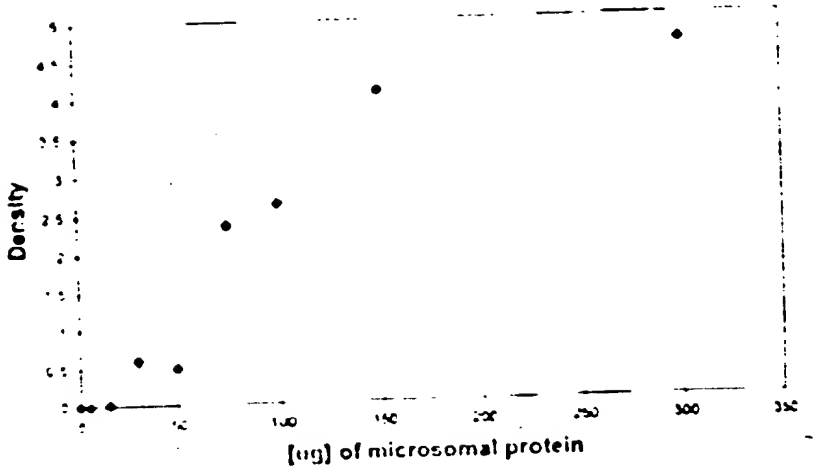

cDNA-Expressed P450 Inhibition by
MAB-2A6 Antibody

(B)


100
80
60
40
20
0
0
10
20
30
40
50
CYP2B1
CYP2B2
Anti-CYP2B1 serum (ul)

FIGURES





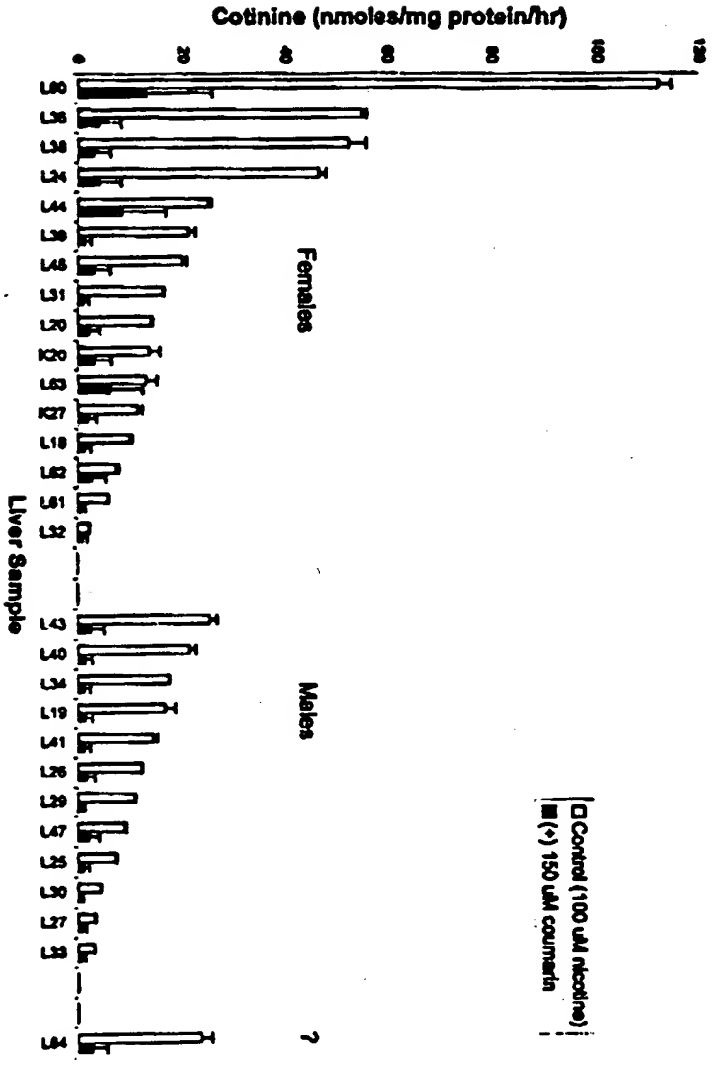

Cotinine (nmoles/mg protein/hr)
0
20
40
60
80
100
120
Females
Males
?
Liver Sample
Control (100 uM nicotine)
(+) 150 uM coumarin

FIGURE 9

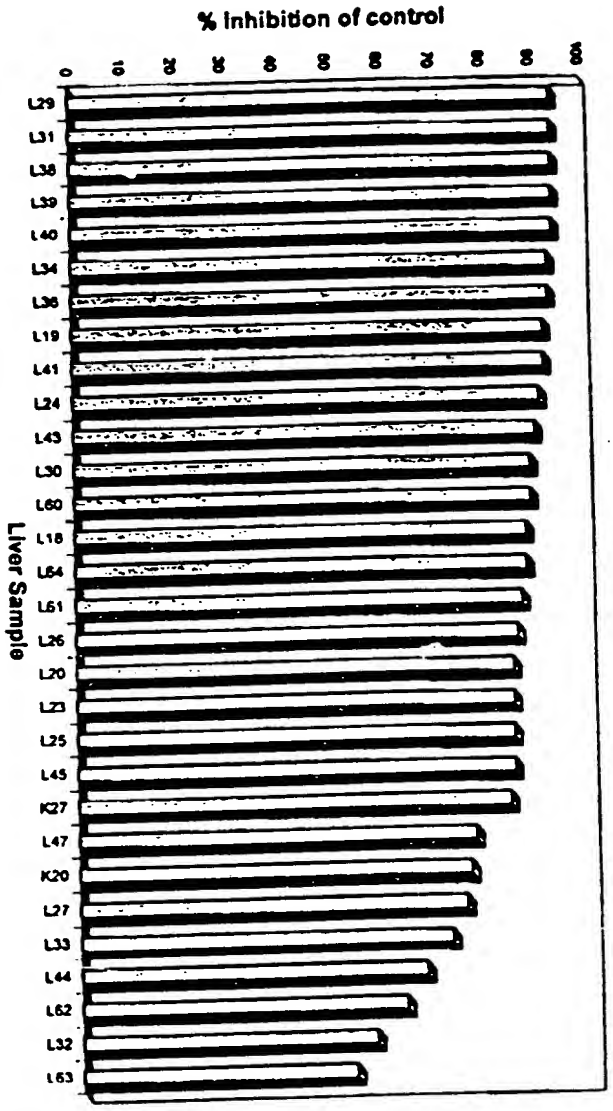
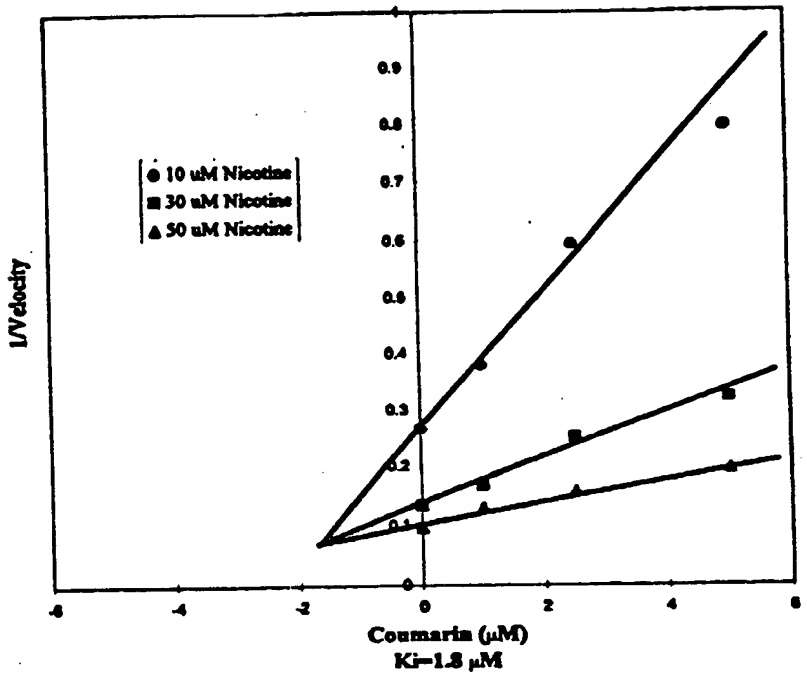
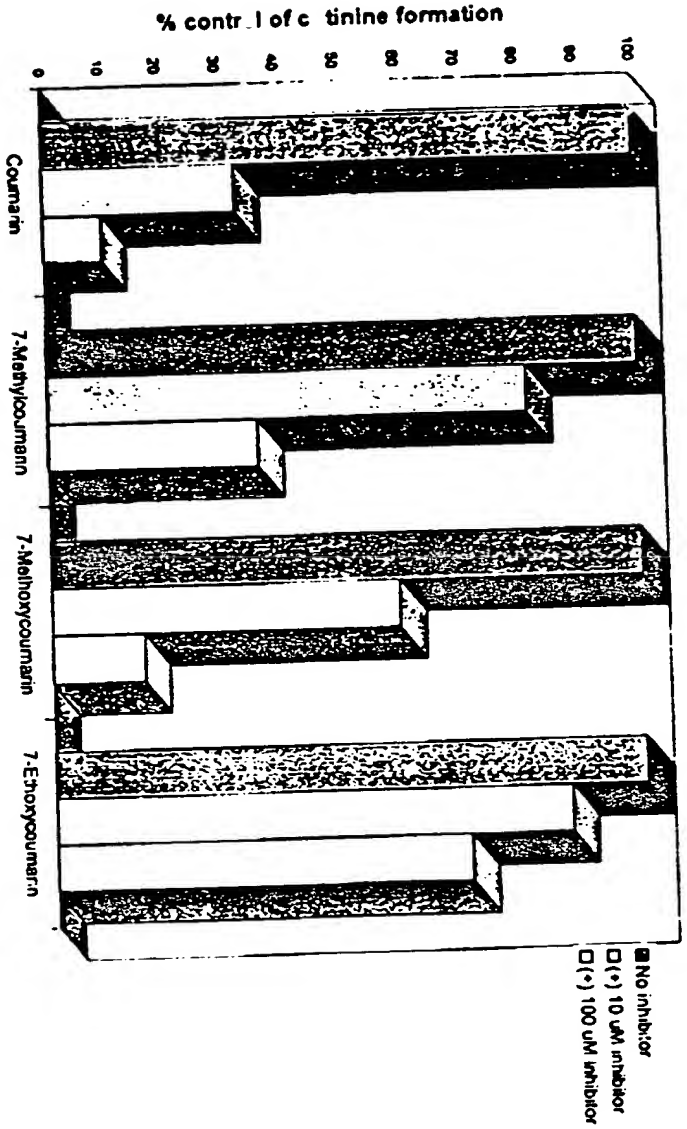

% Inhibition of control
0
10
20
30
40
50
60
70
80
90
100
Liver Sample
L29
L31
L38
L39
L40
L34
L36
L19
L41
L24
L43
L30
L60
L18
L64
L61
L26
L20
L23
L25
L45
K27
L47
K20
L27
L33
L44
L62
L32
L63

FIGURE 10

FIGURE 11


10 uM Nicotine
30 uM Nicotine
50 uM Nicotine
1/Velocity
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-6
-4
-2
0
2
4
6
Coumarin (μM)
Ki=1.8 μM



Rank order of potency in inhibiting cotinine formation:
coumarin > 7-methoxycoumarin > 7-methylcoumarin > 7-ethoxycoumarin

FIGURE 12

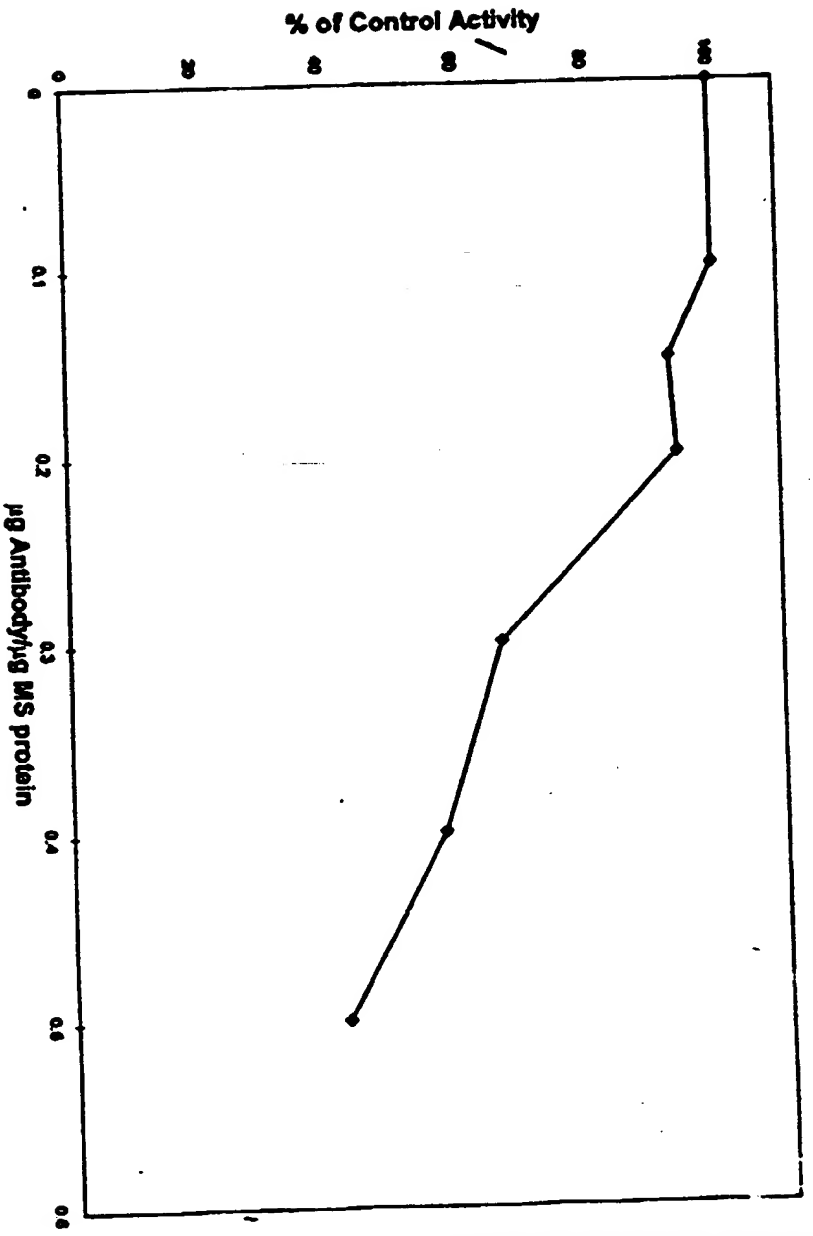


FIGURE 13

FIGURE 14

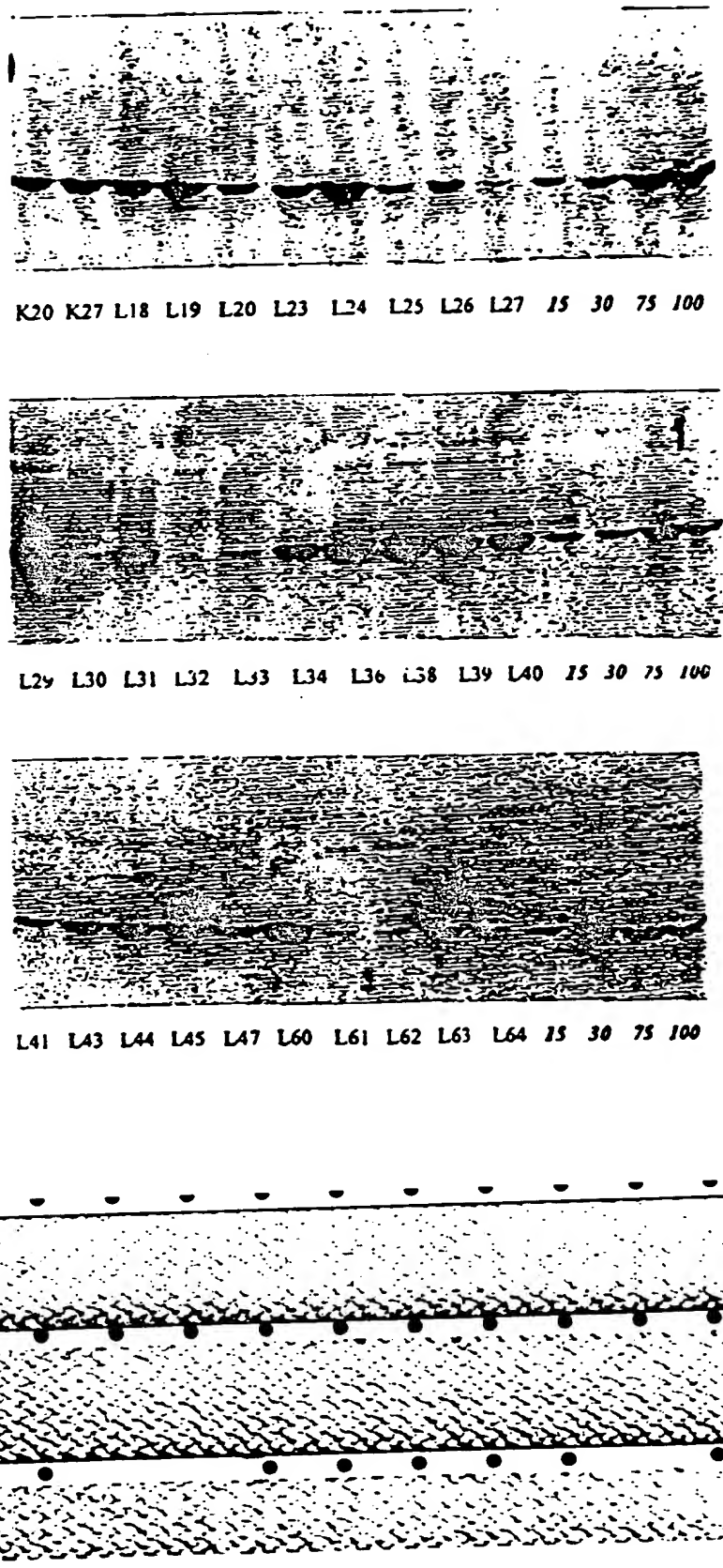
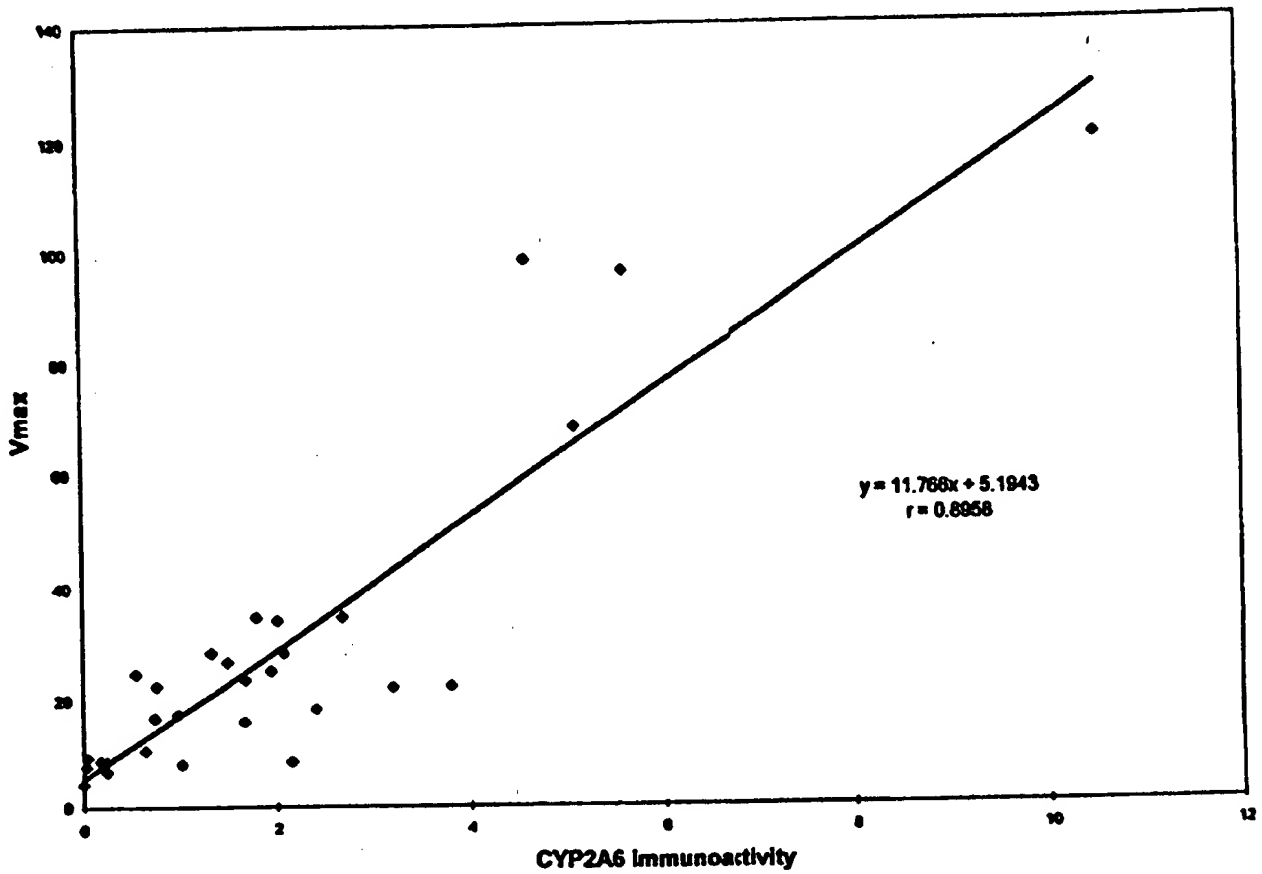

98 kDa
68 kDa
43 kDa
K20 K27 L18 L19 L20 L23 L24 L25 L26 L27 15 30 75 100
L29 L30 L31 L32 L33 L34 L36 L38 L39 L40 15 30 75 100
L41 L43 L44 L45 L47 L60 L61 L62 L63 L64 15 30 75 100

FIGURE 15


Vmax
CYP2A6 Immunoactivity
y = 11.766x + 5.1943
r = 0.8958
0
2
4
6
8
10
12

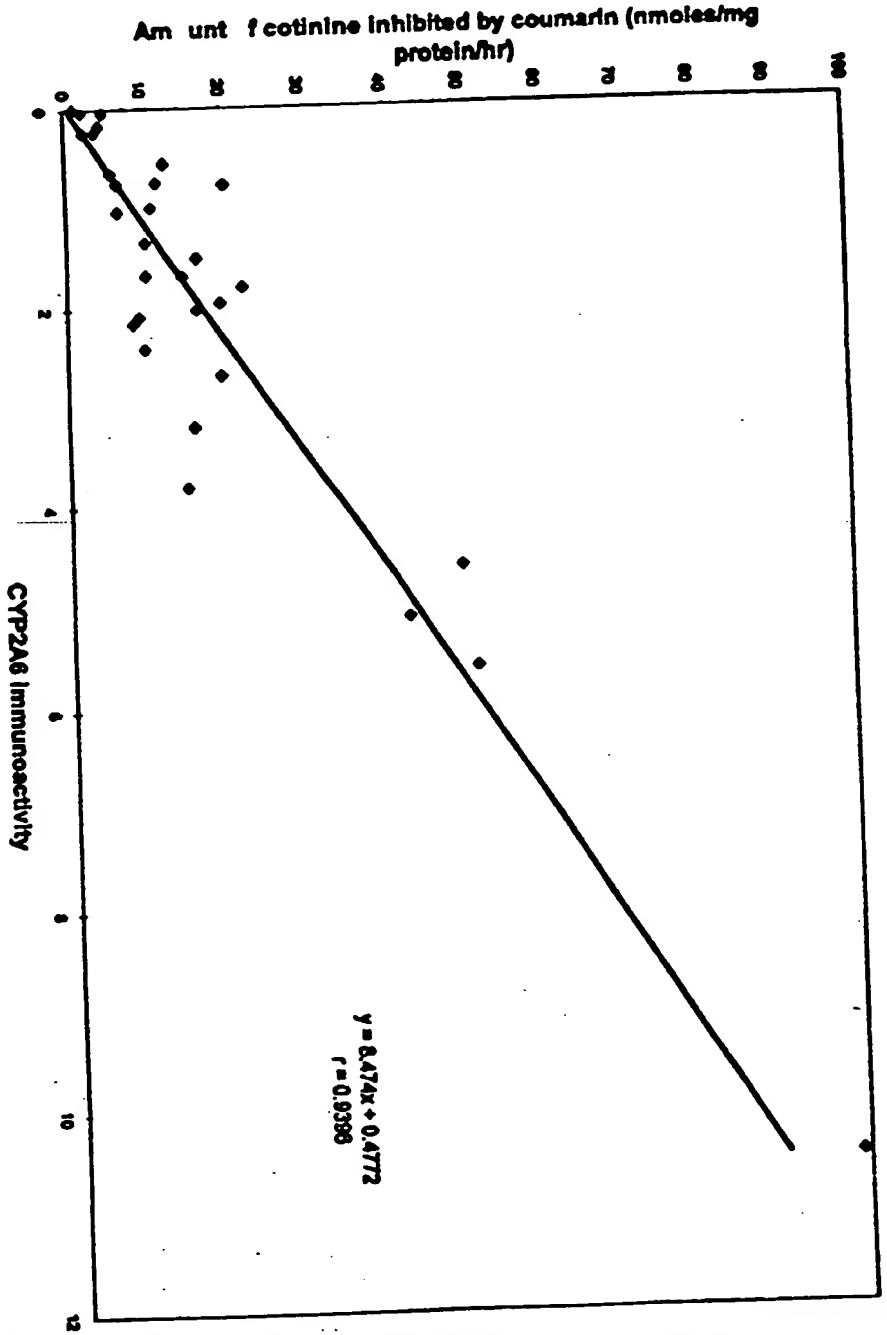

Amount of cotinine inhibited by coumarin (nmoles/mg protein/hr)
CYP2A6 Immunoactivity
y = 8.474x + 0.4772
r = 0.9386

FIGURE 16

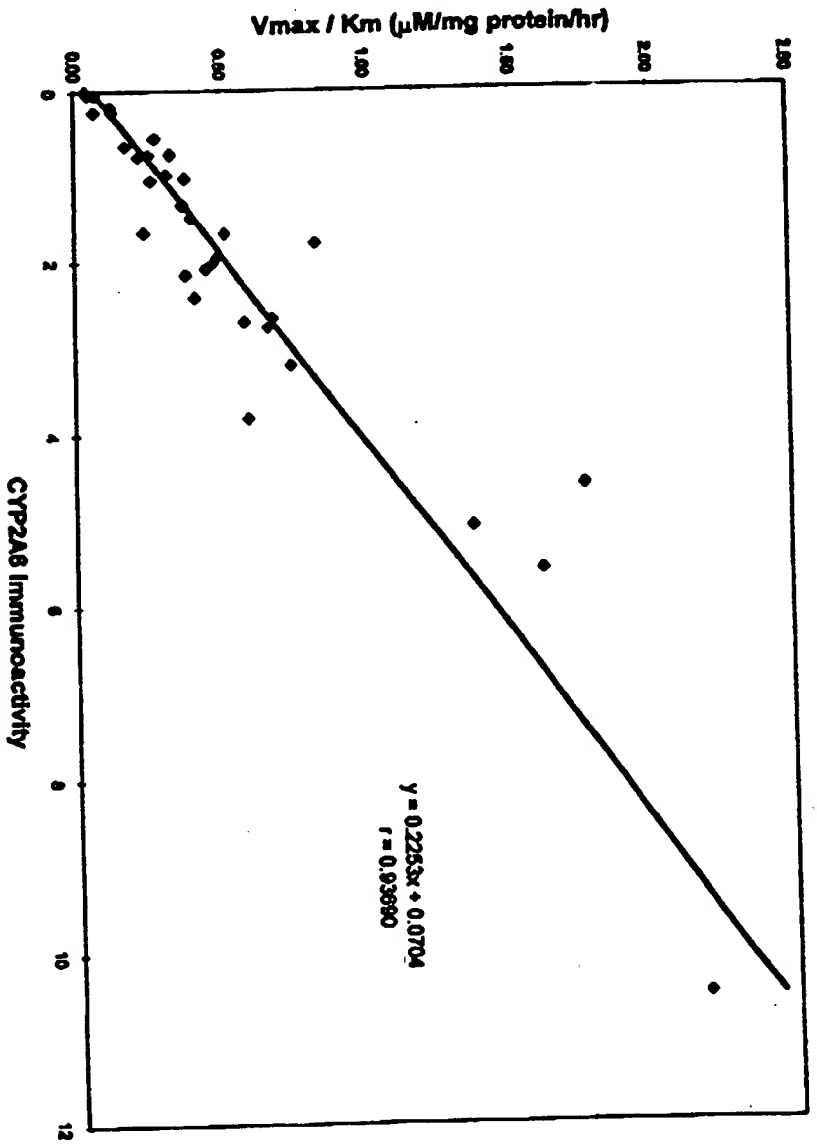

Vmax / Km (µM/mg protein/hr)
0.00
0.50
1.00
1.50
2.00
2.50
0
2
4
6
8
10
12
CYP2A6 Immunoactivity
y = 0.2253x + 0.0704
r = 0.9390

FIGURE 17

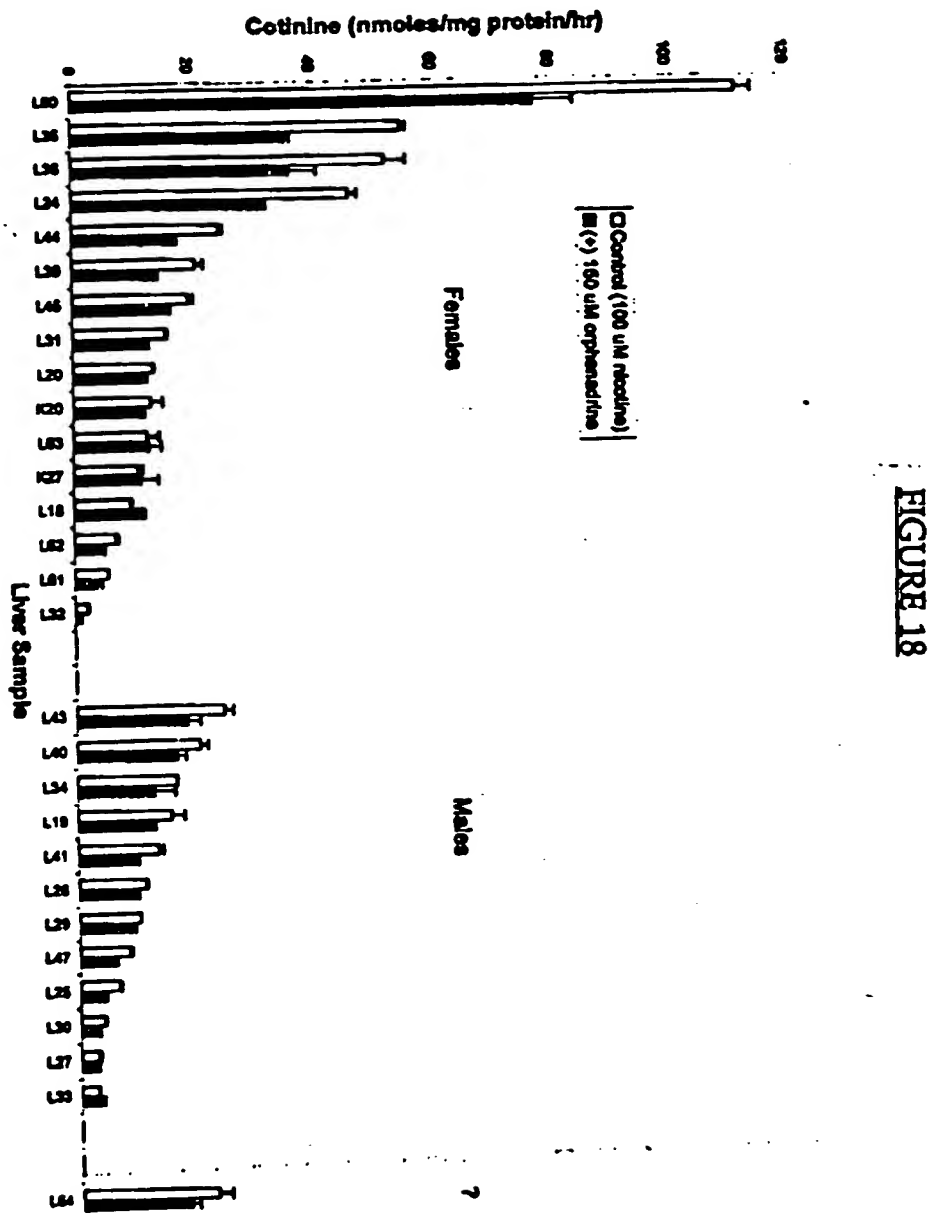

Cotinine (nmoles/mg protein/hr)
Control (100 uM nicotine)
(+) 100 uM orphenadrine
Females
Males
Liver Sample

FIGURE 18

60/021940

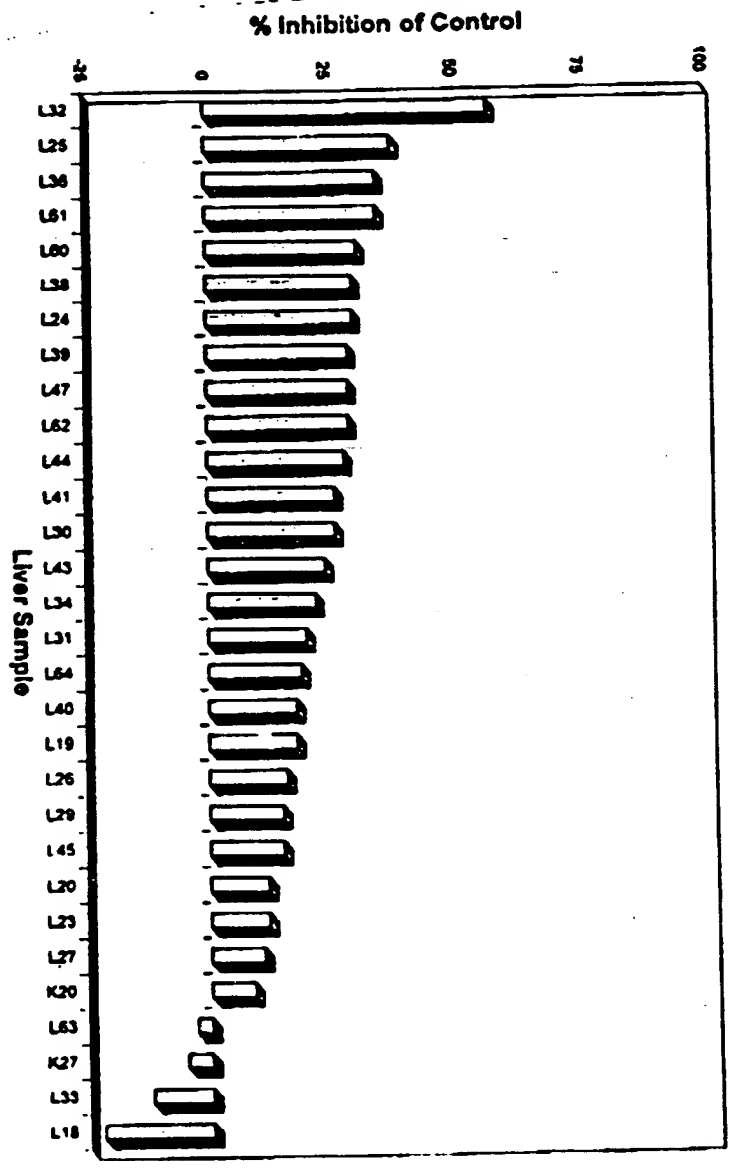


FIGURE 19

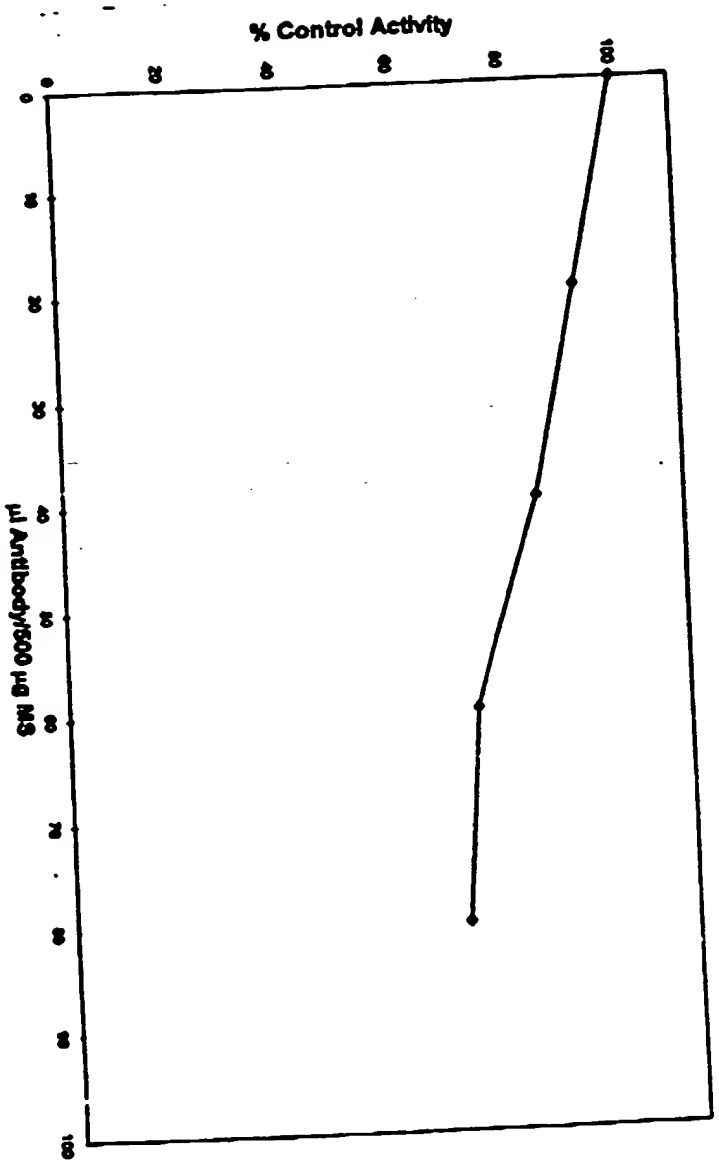


FIGURE 20

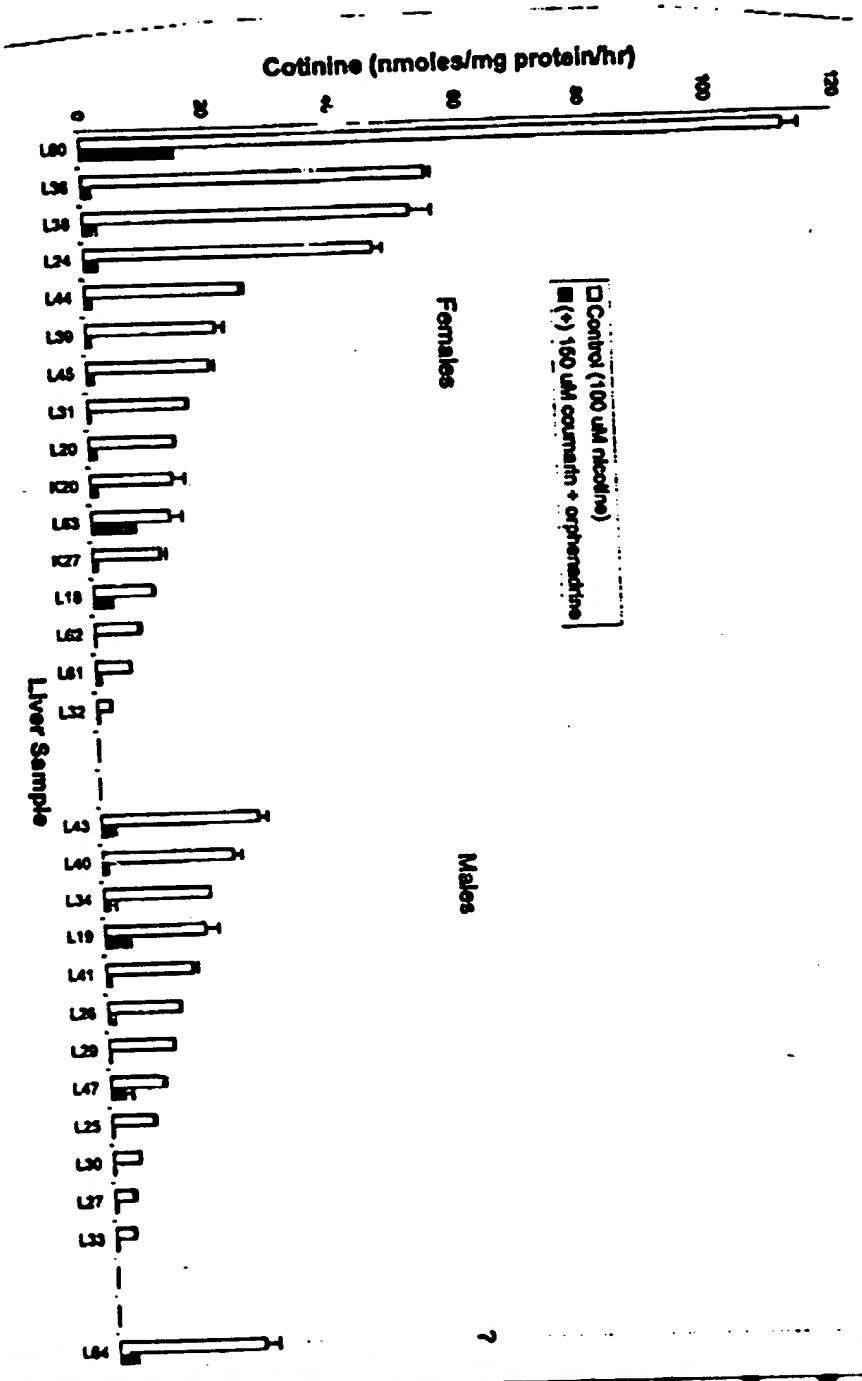

Cotinine (nmoles/mg protein/hr)
0
20
40
60
80
100
120
Females
Males
Liver Sample
L60
L36
L38
L24
L44
L39
L45
L31
L29
L30
L33
L27
L18
L62
L61
L32
L43
L40
L34
L19
L41
L26
L29
L47
L25
L30
L27
L33
L64
Control (100 uM nicotine)
(+) 160 uM coumarin + orphenadrine

FIGURE 21

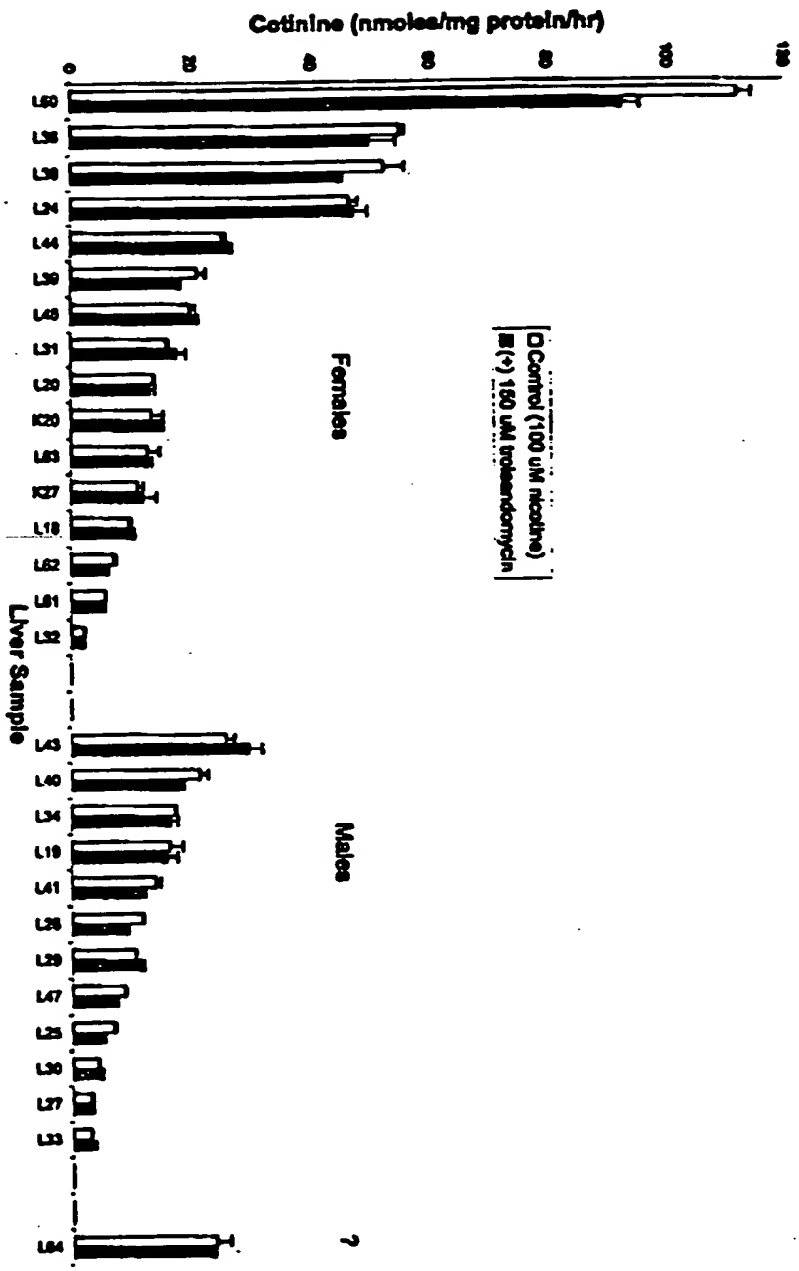
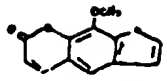
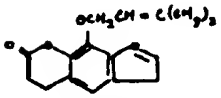

Cotinine (nmoles/mg protein/hr)
0
2
4
6
8
10
Females
Males
Liver Sample
Control (100 uM nicotine)
(+) 150 uM troleandomycin

FIGURE 22

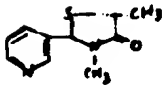
FIGURE 23



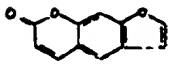
Methoxsalen



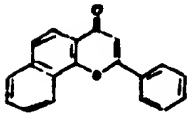
Imperatorin



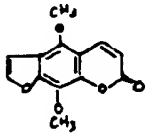
(SM-12502)



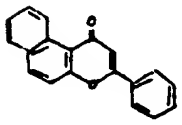
Psoralen



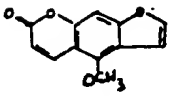
α-Naphthoflavone



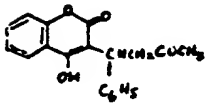
Isopimpinellin



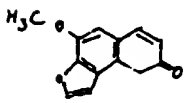
β-Naphthoflavone



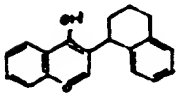
Bergapten



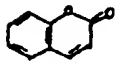
warfarin



Sphondin



Coumatetralyl (Racemic)



coumarin